UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Sanidad Animal

TESIS DOCTORAL

Identificación y caracterización de las proteínas NcROP40 y NcNTPasa, y evaluación de su utilidad vacunal frente a la neosporosis

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid, 2016

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D. Iván Pastor Fernández

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Animal Health Department

Identification and characterization of the NcROP40 and NcNTPase proteins, and assessment of their usefulness as vaccines against neosporosis

DOCTORAL THESIS

Mr. Iván Pastor Fernández

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"Si no hay dudas, no hay progreso".

Charles Darwin (1809 - 1882).

"Observar sin pensar es tan peligroso como pensar sin observar".

Santiago Ramón y Cajal (1852 - 1934).

"Lo importante es no dejar de hacerse preguntas".

Albert Einstein (1879 - 1955).

D. Luis Miguel Ortega Mora, Doctor en Veterinaria y Catedrático de Universidad adscrito al Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid, Dña. Gema Álvarez García, Doctora en Veterinaria y Profesora Titular adscrita al departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid, y D. Javier Regidor Cerrillo, Doctor en Farmacia e Investigador post-doctoral adscrito al Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid

CERTIFICAN:

1) Que la tesis doctoral titulada **"Identificación y caracterización de las proteínas NcROP40 y NcNTPasa, y evaluación de su utilidad vacunal frente a la neosporosis"** presentada por el Licenciado en Veterinaria D. Iván Pastor Fernández para optar al grado de Doctor por la Universidad Complutense de Madrid ha sido realizada en las dependencias del Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid bajo su supervisión.

2) Que la presente tesis doctoral cumple con todas las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid con Mención Europea.

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En Madrid, a 29 de octubre de 2015.

Dr. Luis Miguel Ortega Mora Dra. Gema Álvarez García Dr. Javier Regidor Cerrillo

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La presente tesis doctoral cumple con los requisitos exigidos por la Universidad Complutense de Madrid para obtener la mención de Doctor europeo:

1) Estancias en centros de investigación europeos:

- Centro receptor: Karolinska Institutet, Center for Infectious Medicine (Stockholm, Sweden).
- Investigador principal: Dr. Antonio Barragán.
- Duración de la estancia: 4 meses (01/04/2013 - 31/07/2013).

2) Evaluadores externos europeos:

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- Dr. Antonio Barragán. Institutionen för Molekylär biovetenskap, Wenner-Grens Institut, Stockholms Universitet (Stockholm, Sverige).

3) Miembros europeos del tribunal evaluador de la tesis doctoral:

- Dr. Andrew Hemphill. Universität Bern, Institut für Parasitologie (Bern, Schweiz).

"Toca afinar, definir de un trazo, sintonizar, reagrupar pedazos..."

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ABBREVIATIONS INDEX

1 or 2-DE:	1 or 2-Dimensional Electrophoresis
6PGD:	6-Phosphogluconate Dehydrogenase
aa:	aminoacid
ACT:	Actin protein
AMA:	Apical Membrane Antigen
AspRS:	Aspartyl-tRNA Synthetase
BAG:	Bradyzoite Antigen
BSA:	Bovine Serum Albumin
BSR:	Bradyzoite Surface Antigen
BVD:	Bovine Virus Diarrhea
CBB:	Coomasie Brilliant Blue
CD4+/8+:	Cluster of Differentiation 4+/8+
CIP:	Alkaline Phosphatase, Calf Intestinal
CPDK:	Calcium-Dependent Protein Kinase
Cyt.D:	Cytochalasin D
DIA:	Differential In-gel Analysis
DIGE:	Difference Gel Electrophoresis
DIVA:	Differentiating Infected from Vaccinated Animals
DTT:	Dithiothreitol
EC:	Enzyme Comision number
EDA:	Extended Data Analysis
EST:	Expressed Sequence Tag
EtOH:	Ethanol
G6PD:	Glucose-6-Phosphate Dehydrogenase
GAP:	Gliding Associated Protein
GRA:	Dense granule protein
HFF:	Human Foreskin Fibroblast
hpi:	hours post-infection
HRP:	Horseradish Peroxidase
HSP:	Heat Shock Protein
IBR:	Infectious Bovine Rhinotracheitis
IDA:	Immunodominant Antigen
IEF:	Isoelectric Focusing
IFN-γ:	Interferon gamma
IgG:	Immunoglobulin G
IL:	Interleukin
IMAC:	Immobilized Metal ion Affinity Chromatography
IRG:	Immunity Related GTPase
ISCOM:	Immune Stimulating Complexes
ITS:	Internal Transcribed Spacer
LC:	Liquid Chromatography
MAb:	Monoclonal Antibody
MALDI-TOF:	Matrix-Assisted Laser Desorption/Ionization – Time Of Flight
MeOH:	Methanol
MIC:	Microneme protein
MLC:	Myosin Light Chain protein
MOI:	Multiplicity Of Infection
MPSS:	Massively Parallel Signature Sequencing
MS:	Mass Spectrometry
MW/Mr:	Molecular Weight/ Relative Molecular Mass
NF-κB:	Nuclear factor kB
NK(-T):	Natural Killer (T Cell)
NO:	Nitric oxide
NTP(D)ase:	Nucleoside Triphosphate (diphospho)hydrolase
O.D.:	Optical Density
ORF:	Open Reading Frame
p.c.:	post-challenge
p.p.:	post-partum
PAb:	Polyclonal Antibody
PDI:	Protein Disulfide Isomerase
PFA:	Paraformaldehyde
PFA+GA:	Paraformaldehyde + Glutaraldehyde
PI:	Phosphatase Inhibitor
PPP:	Pentose Phosphate Pathway
PV(M):	Parasitophorous Vacuole (Membrane)
RAH:	Arginine-rich Amphipathic HelIx Domain
RAPD:	Random Amplification of Polymorphic DNA
RON:	Rhoptry neck protein
ROP:	Rhoptry bulb protein
ROPK:	Rhoptry Kinase
S.D.:	Standard Deviation
SAG:	Surface Antigen
SAGE:	Serial Analysis of Gene Expression
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SNP:	Single Nucleotide Polymorphism
SRS:	SAG1-Related Sequence
SUB:	Subtilisin-like protein
TEM:	Transmission Electron Microscopy
TGF-β:	Transforming Growth Factor beta
Th:	T helper cell
TLR:	Toll-like Receptor
TNF-α:	Tumor Necrosis Factor alpha
TSS:	Transcription Start Site
TT:	Transplacental Transmission
TUBα:	Tubulin alpha protein
VDAC:	Voltage Dependent Anion Channel
WB:	Western blot

RESUMEN/ABSTRACT

Neospora caninum es un parásito formador de quistes reconocido a nivel mundial como la principal causa de aborto en ganado vacuno, donde produce importantes pérdidas económicas. A pesar de que la vacunación se ha descrito como la estrategia de control más eficiente frente a la neosporosis bovina, hasta la fecha no existen formulaciones eficaces que prevengan la transmisión vertical y el aborto. En la actualidad, las medidas de control de la enfermedad dependen de un diagnóstico adecuado asociado a unas medidas de manejo concretas, por lo que el desarrollo de una vacuna frente a la neosporosis bovina es una tarea urgente. En este sentido, las vacunas de subunidades se presentan como una buena alternativa al uso de vacunas vivas, debido a su mayor seguridad y menor coste de producción; además, dichas vacunas pueden ser específicamente diseñadas frente a proteínas determinadas con el fin de bloquear procesos esenciales para la supervivencia del parásito. Desgraciadamente, el conocimiento de los mecanismos de invasión y proliferación de *N. caninum* es muy limitado a nivel molecular. Además, son pocos los estudios en los que se haya abarcado la identificación de factores de virulencia potenciales del parásito. Todo ello dificulta la selección de dianas apropiadas a la hora de diseñar nuevas formulaciones vacunales frente a la neosporosis.

El primer objetivo de la presente tesis doctoral fue determinar los cambios en el proteoma e inmunoma de dos aislados virulentos (Nc-Liv y Nc-Spain7) y uno no virulento (Nc-Spain1H) mediante DIGE y Western-blot en dos dimensiones. La hipótesis de partida fue que las diferencias en la abundancia de proteínas entre los distintos aislados permitirían identificar nuevos candidatos vacunales. Los estudios de DIGE acoplados a las técnicas de MALDI TOF-MS determinaron que el aislado Nc-Spain7 presenta el mayor número de proteínas diferencialmente expresadas con respecto a los aislados Nc-Liv y Nc-Spain1H. Sin embargo, no se encontró ningún punto proteico específico de ningún aislado. En este sentido, las proteínas NcMIC1, NcROP40, NcNTPasa, glucosa-6-fosfato deshidrogenasa y aspartil-ARNt sintetasa mostraron una mayor abundancia en los aislados virulentos. Estas variaciones podrían determinar las diferencias en el comportamiento *in vitro* e *in vivo* entre los aislados estudiados, aportando candidatos vacunales potenciales para desarrollar vacunas de subunidades. Por otro lado, y de forma complementaria, se estudiaron los posibles cambios existentes en el perfil antigénico de los aislados Nc-Liv, Nc-Spain7 y Nc-Spain1H. Para ello, se utilizaron sueros de ratones experimentalmente infectados con cada uno de los tres aislados, y se enfrentaron individualmente a cada uno de los tres extractos proteicos de cada aislado (diseño 3x3). Curiosamente, las diferencias en la abundancia de proteínas entre los aislados estudiados no tuvieron influencia alguna en su perfil antigénico, que fue muy similar entre ellos. Las principales variaciones observadas en su inmunoma dependieron únicamente del suero empleado. El suero de los ratones infectados con el aislado Nc-Liv reconoció el mayor número de antígenos, mientras que el suero de los ratones infectados con el aislado Nc-Spain1H detectó un menor número. Estas diferencias podrían ser debidas a las variaciones en la dinámica de infección descritas entre los distintos aislados. Destacablemente, el suero de los ratones infectados con el aislado Nc-Spain1H no reconoció a las proteínas NcGAP45, serín-treonín fosfatasa 2C, superóxido dismutasa y NcGRA1, lo que sugiere su uso potencial como marcadores de viurlencia.

El segundo objetivo de la presente tesis doctoral fue dirigido a caracterizar aquellas proteínas más abundantes en los aislados virulentos, exclusivamente encontradas en las organelas de secreción de los parásitos apicomplejos y escasamente estudiadas hasta la fecha. De acuerdo a estos criterios, las proteínas NcROP40 y NcNTPasa fueron caracterizadas por primera vez. Las secuencias de los genes que codifican para NcROP40 y NcNTPasa fueron analizadas en detalle. Posteriormente, ambas se analizaron en el contexto de las relaciones parásito-hospedador y en comparación con las proteínas NcROP2Fam-1 y NcGRA7, previamente caracterizadas. Para ello, se llevó a cabo el estudio de la dinánima de las proteínas, su secreción, fosforilación y perfil de transcripción a lo largo del ciclo lítico del taquizoíto.

Los análisis de la secuencia ORF disponible para Nc*ROP40* evidenciaron que su extremo N-terminal no estaba correctamente anotado, lo que dio lugar a a descripción de la secuencia Nc*ROP40-long*, la versión corregida de la anotación inicial de Nc*ROP40*. Curiosamente, la comparación del ORF de Nc*ROP40* de los aislados Nc-Liv, Nc-Spain7 y Nc-Spain1H no reveló ningún polimorfismo que pudiera explicar las diferencias en la abundancia y en la virulencia entre ellos. Además, la proteína carece de los dominios RAH, lo que explica su falta de interacción con la membrana de la vacuola parasitófora (MVP) mediante inmunofluorescencia. NcROP40 se localiza en el cuerpo de las roptrias, siendo probablemente objeto de una maduración proteolítica, aunque su secreción no fue evidenciada. No obstante, NcROP40 podría secretarse en el citosol de la célula hospedadora o ser translocada a su núcleo, donde podría alterar la expresión génica del hospedador. En contraste, la proteína NcROP2Fam-1 se secreta ampliamente y se asocia con la MVP. Curiosamente, la descarga de las roptrias no se vio afectada por un incremento de los niveles de calcio intracelular, y la secreción de NcROP2Fam-1 sólo se asoció al contacto con la célula hospedadora.

Por otro lado, en el presente trabajo se ha descrito por primera vez la distribución genómica de la proteína NcNTPasa. Ésta se encuentra codificada por tres *loci* diferentes que contienen tres copias distintas del gen dentro del genoma del aislado Nc-Liv. Además, se ha demostrado la existencia de hasta seis alelos diferentes para la proteína. Las proteínas NcNTPasa y NcGRA7 fueron establecidas como proteínas GRA canónicas, cuya secreción es detectable durante la invasión temperana, la formación de la vacuola parasitófora y la egresión. Ambas mostraron una interacción clara con la MVP, aunque se evidenciaron diferencias en su distribución durante la invasión y la egresión. Además, la secreción de las proteínas NcNTPasa y NcGRA7 demostró ser un proceso regulado de forma diferente entre ambas, siendo la secreción de la primera independiente de las cascadas intracelulares de calcio.

Asímismo, la expresión de ARNm de las proteínas NcROP40, NcROP2Fam-1, NcNTPasa y NcGRA7 mostró un perfil similar a lo largo del ciclo lítico. Todos los transcritos alcanzaron un pico de expresión a las 6 y 56 horas post-infección, coincidiendo con la invasión temprana y/o la egresión. Por lo tanto, su función podría ser necesaria para garantizar la progresión del ciclo lítico en las fases siguientes del mismo. No obstante, sólo la expresión de NcROP2Fam-1, NcNTPasa y NcGRA7 se vio afectada por la

suplementación con DTT, lo que sugiere su participación más directa en la egresión. Además, sólo las proteínas NcROP2Fam-1, NcNTPasa y NcGRA7 fueron fosforiladas durante esta etapa, coincidiendo con sus niveles máximos de transcripción y con la secreción de la proteína. Estos hallazgos podrían indicar un mecanismo de regulación común necesario para su participación en el ciclo lítico.

El tercer objetivo de la presente tesis doctoral fue evaluar la utilidad vacunal de las proteínas NcROP40, NcROP2Fam-1, NcNTPasa y NcGRA7 formuladas como preparados polivalentes en un modelo murino de neosporosis bien establecido. Todas las formulaciones redujeron la gravedad de la infección por *N. caninum* en los ratones vacunados, aunque las cargas parasitarias fueron menos pronuciadas en los ratones vacunados con las proteínas de roptrias. La vacunación prolongó ligeramente el tiempo de supervivencia medio de las crías, aunque sólo rNcROP2Fam-1 y la combinación de rNcROP40 con rNcROP2Fam-1 previnieron la mortalidad neonatal, al menos parcialmente, con tasas de supervivencia del 6.3 y del 16.2%, respectivamente. En este sentido, cabe destacar que la combinación de rNcROP40 con rNcROP2Fam-1 mostró un efecto sinérgico y bloqueó la transmisión vertical en todas las crías supervivientes y procedentes de diferentes camadas. Por el contrario, no se observó ningún sinergismo tras la asociación de las proteínas rNcNTPasa y rNcGRA7. Por lo tanto, el uso de proteínas de roptrias como antígenos en formulaciones vacunales e presenta como una estrategia prometedora para bloquear los procesos en los que éstas participan durante el ciclo lítico del parásito.

La presente tesis doctoral abarcó la identificación de aquellos elementos diferencialmente abundantes etre aislados virulentos y no virulentos con el fin de profundizar en los mecanismos subyacentes a la virulencia del parásito. Las proteínas NcROP40, NcROP2Fam-1, NcNTPasa y NcGRA7 han mostrado interesantes características que están asociadas a fases específicas del ciclo lítico, por lo que parecen ser esenciales para su progresión. Además, este trabajo sugiere el uso potencial de las proteínas de roptrias como antígenos para el desarrollo de futuras formulacioes vacunales. Sin embargo, y dado que hasta la fecha no se ha identificado ningún factor de virulencia de *N. caninum*, sería necesario estudiar la relevancia funcional de las proteínas de la familia ROP2 en la virulencia del parásito y en su probable modulación de la célula hospedadora. Por otro lado, se requieren más estudios para aclarar el papel de la NcNTPasa en la patogenicidad del parásito. En este escenario, la utilización de técnicas de genética reversa sería de gran utilidad para dilucidar la función de las proteínas NcROP40 y NcNTPasa dentro del ciclo lítico. Además, sería necesario realizar experimentos a gran escala que incluyeran un mayor número de aislados de *N. caninum*. Para ello, el empleo de plataformas proteómicas complementarias y la disponibilidad de un mayor número de genomas secuenciados de distintos aislados del parásito serían de gran utilidad para identificar nuevos elementos implicados en la patogenicidad del parásito.

Neospora caninum is a cyst-forming parasite that has been recognized worldwide as the main cause of abortion in cattle producing significant economic losses. Despite vaccination having been described as the most cost-efficient strategy to control neosporosis in cattle, there are no available formulations to prevent vertical transmission and abortion. Nowadays, control options rely on diagnosis coupled with management measures, and hence, the development of an effective vaccine against bovine neosporosis has become urgent. Subunit vaccines appear as a good alternative to the use of live vaccines due to their safety and reduced costs. In addition, they can be specifically designed to act against particular proteins to block essential processes for parasite survival. Unfortunately, the basic mechanisms on how host cell invasion and proliferation is achieved by *N. caninum* tachyzoites have been poorly investigated at the molecular level. Moreover, the identification of potential virulence factors of *N. caninum* has not been extensively exploited, making the choice of proper targets to design new vaccine formulations against neosporosis difficult.

The first objective of the present thesis was to determine the proteome and immunome changes among two virulent (Nc-Liv and Nc-Spain7) and one non-virulent (Nc-Spain1H) well-characterized *N. caninum* isolates by DIGE and 2-DE immunoblot techniques. We hypothesize that proteins differentially abundant among them would be valuable vaccine candidates against bovine neosporosis. When pairwise comparisons of the isolates proteomes were done by DIGE coupled to MALDI TOF-MS, the Nc-Spain7 isolate showed the highest number of differentially abundant spots with respect to the Nc-Liv and the Nc-Spain1H. However, no specific-isolate spots were found. In this sense, the NcMIC1, NcROP40, NcNTPase, glucose-6-phosphate dehydrogenase and aspartyl-tRNA synthetase proteins showed to be more abundant in virulent isolates. These variations may determine the differences in the *in vivo* and *in vitro* behaviour among isolates, and provide potential candidates to develop subunit vaccines against *N. caninum*. As a complementary approach, immunome changes among these isolates were also analysed. Sera from mice infected with each one of the three isolates were exposed individually to protein extracts of each one of the isolates (3x3 design). Interestingly, differences in protein abundance did not influence the antigenic profiles of the three isolates, which were very similar among them. The major differences observed in the immunome pattern were uniquely dependent on the pool of sera used, which is likely due to variations in the infection dynamics among isolates. Sera from mice experimentally infected with Nc-Liv was seen to recognize the highest number of antigens, whilst sera from mice experimentally infected with Nc-Spain1H achieved the lowest spot recognition. Remarkably, the lack of recognition of the NcGAP45, serine-threonine phosphatase 2C, superoxide dismutase, and NcGRA1 proteins by the Nc-Spain1H sera suggests their potential use as virulence markers.

The second objective of the present doctoral thesis aimed to characterize those proteins more abundant in virulent isolates, exclusively found in apicomplexan parasites from secretory organelles, and scarcely investigated to date. According to these criteria, the NcROP40 and NcNTPase proteins were characterized for the first time. Protein gene sequences were analysed in detail. Both proteins were then

compared with the previously characterized NcROP2Fam-1 and NcGRA7, respectively, and analysed in the context of the host-parasite relationships through a descriptive approach that included the study of the protein dynamics, secretion, phosphorylation, and transcript expression profile throughout the tachyzoite lytic cycle.

Nc*ROP40* sequence analyses evidenced the lack of an annotated N-terminus on its ORF, and lead to the description of the Nc*ROP40*-long sequence, a corrected annotation of the Nc*ROP40* gene. Interestingly, comparison of the Nc*ROP40* ORF among Nc-Liv, Nc-Spain7 and Nc-Spain1H isolates did not reveal any polymorphism that could explain the differences in abundance and virulence among them. Moreover, the protein lacks the RAH domains, that may explain why the protein does not interact with the parasitophorous vacuole membrane (PVM) as evidenced by immunofluorescence analyses. NcROP40 is localized in rhoptry bulbs and is likely subject to proteolytic maturation, but no evidence for NcROP40 secretion was observed. Nevertheless, NcROP40 could be secreted into the host-cell cytosol or be translocated to host nucleus and disturb the gene expression of the host cell. In contrast, NcROP2Fam-1 showed to be extensively secreted and associated with the PVM. Intriguingly, rhoptry discharge was not affected by elevated intracellular calcium levels, and NcROP2Fam-1 secretion was only induced upon host cell contact.

On the other hand, we described for the first time the genome distribution of the NcNTPase protein. The protein is coded by three different *loci* containing three different copies of the Nc*NTPase* gene within the Nc-Liv genome. Moreover, the existence of up to six different Nc*NTPase* alleles has been also shown. The NcNTPase and NcGRA7 proteins were established as canonical GRA proteins, whose secretion was detected during early invasion, parasitophorous vacuole formation, and egress. Both proteins exhibited a clear interaction with the PVM, but displayed differences in their distribution during invasion and egress. Furthermore, NcNTPase and NcGRA7 secretion occurred in tachyzoites undergoing egress under different mechanisms of regulation, since NcNTPase release was independent of the intracellular calcium cascades.

Furthermore, mRNA expression of the NcROP40, NcROP2Fam-1, NcNTPase and NcGRA7 showed a similar profile throughout the lytic cycle. The transcripts reached a peak at 6 and 56 hours post-infection, coinciding with egress and/or early invasion. Therefore, their function may be necessary to guarantee the lytic cycle progression in subsequent lytic cycle phases. Nevertheless, only NcROP2Fam-1, NcNTPase and NcGRA7 expression was affected by DTT supplementation, which is suggestive of their involvement in egress. Moreover, NcROP2Fam-1, NcGRA7 and NcNTPase became phosphorylated at this stage, coinciding with the maximum transcript levels and with the protein release. These observations might indicate a common regulation mechanism necessary for their participation within the lytic cycle.

The third objective of the present doctoral thesis was to evaluate the usefulness of polyvalent formulations based on the NcROP40, NcROP2Fam-1, NcGRA7 and NcNTPase proteins in a well-established mouse model of neosporosis. All formulations reduced the severity of *N. caninum* infection in vaccinated mice, although parasite burdens were less pronounced on mice vaccinated with rhoptry proteins. Vaccination slightly prolonged the median survival time of pups, but only rNcROP2Fam-1 and rNcROP40+rNcROP2Fam-1 prevented pup mortality, at least partially, with survival rates of 6.3 and 16.2%, respectively. Remarkably, the rNcROP40+rNcROP2Fam-1-based formulation showed a synergistic effect and blocked vertical transmission in all surviving pups from different litters. Conversely, no synergism was achieved by combining rNcGRA7 and rNcNTPase proteins. Hence, the use of rhoptry proteins as antigens in vaccine formulations appears to be a promising strategy to block the processes in which they are involved.

The present thesis aimed to identify those elements differentially abundant among virulent and non-virulent isolates in order to deepen the understanding of the mechanisms underlying parasite virulence. NcROP40, NcROP2Fam-1, NcNTPase, and NcGRA7 proteins have shown interesting features associated with specific stages of the lytic cycle, and thus, they are likely relevant for its progression. Furthermore, our study suggests the potential use of rhoptry proteins as antigens in vaccine formulations. Nevertheless, future investigations are required to establish the functional relevance of ROP2-family proteins in host-cell modulation and virulence, since no *N. caninum* virulence factors have been identified to date. In addition, further studies must be carried out in order to clarify the impact of the NcNTPase expression on parasite pathogenicity. In this scenario, the employment of reverse genetics approaches would be useful to elucidate the role of the NcROP40 and NcNTPase proteins within the lytic cycle. Moreover, large-scale experiments including a higher number of *N. caninum* isolates, complementary proteomic platforms and the availability of different genome annotations from diverse *N. caninum* isolates would be helpful to identify new elements implicated in the parasite pathogenicity.

INTRODUCCIÓN

1. *Neospora caninum* y neosporosis bovina

Neospora caninum es un protozoo apicomplejo identificado como el agente causal de la neosporosis bovina. Dicha enfermedad se considera una de las principales causas de aborto en el ganado bovino a nivel mundial (Dubey & Schares, 2011). El parásito desarrolla un ciclo biológico heteroxeno en sus hospedadores, siendo el perro y el ganado vacuno los de mayor importancia. El perro actúa como hospedador definitivo e intermediario, mientras que los bovinos únicamente actúan como hospedadores intermediarios. El primer caso de neosporosis fue documentado en Noruega (Bjerkås *et al.*, 1984), donde se describió un cuadro clínico de encefalomielitis y miositis graves en un perro infectado por un parásito formador de quistes que no había sido identificado hasta el momento. Posteriormente, la presencia de este mismo agente fue asociada a casos de encefalomielitis en terneros recién nacidos (O'Toole & Jeffrey, 1987; Parish *et al.*, 1987). La propuesta del género *Neospora* y de la especie *N. caninum* se remonta a 1988, cuando el parásito fue aislado por primera vez (Dubey *et al.*, 1988). Un año después se describió a *N. caninum* como agente causal de aborto bovino (Thilsted & Dubey, 1989). Dado que las diferencias estructurales entre géneros del mismo taxón son escasas, muchos estudios previos a 1984 pudieron atribuir erróneamente ciertos casos clínicos producidos por *Neospora* a otros géneros cercano filogenéticamente tales como *Toxoplasma* (Bjerkås & Dubey, 1991) y *Hammondia* (Dubey *et al.*, 2002b; McAllister, 2000; Mehlhorn & Heydorn, 2000). Actualmente, la relevancia de la enfermedad es mayor en el ganado bovino, donde se considera una de las principales causas de fallo reproductivo de etiología transmisible a nivel mundial (Dubey & Schares, 2011). Hasta el momento no existen tratamientos ni vacunas eficaces frente a la infección, y su control se basa en medidas de manejo de los rebaños afectados (Reichel *et al.*, 2014). A nivel mundial, las pérdidas económicas debidas a la neosporosis bovina en las principales potencias ganaderas se han estimado en un billón de dólares (Reichel *et al.*, 2013).

1.1. Clasificación taxonómica y ultraestructura del parásito

Neospora caninum es un protozoo parásito intracelular obligado y clasificado dentro del phylum Apicomplexa, que incluye a más de 6.000 especies que parasitan prácticamente a todos los grupos de animales y que se caracterizan por la presencia de un complejo apical (Adl *et al.*, 2007). Más concretamente, *N. caninum* se encuadra dentro de la familia Sarcocystidae/subfamilia Toxoplasmatinae por su capacidad para formar quistes tisulares en los hospedadores parasitados, por la presencia de ciclos biológicos heteroxenos con alternancia de fases de replicación sexual y asexual en sus hospedadores y por la capacidad de esporulación de los ooquistes en el medio ambiente (Tenter *et al.*, 2002). La subfamilia Toxoplasmatinae incluye además los géneros *Toxoplasma*, de gran importancia en medicina humana y en veterinaria (Schluter *et al.*, 2014) y *Besnoitia*, con interés emergente en medicina veterinaria (Álvarez-García *et al.*, 2013a). Dentro del género *Neospora* se conocen dos especies: *N. caninum*, que afecta principalmente a los ungulados (Dubey *et al.*, 1988) y *N. hughesi*, que afecta a los

équidos (Marsh *et al.*, 1998). No obstante, esta última especie ha sido menos estudiada que *N. caninum* (Howe *et al.*, 2014).

Hasta el momento se han descrito tres estadios invasivos en el ciclo biológico de *N. caninum*: los taquizoítos, los bradizoítos y los esporozoítos (Figura 1). Los taquizoítos constituyen el estadio parasitario mejor estudiado por su rápida replicación y facilidad de obtención *in vitro*. Éstos son los responsables de la fase aguda de la enfermedad, ya que invaden de forma activa a las células hospedadoras y proliferan de forma masiva en su interior. De tal modo, la sucesión de ciclos de replicación intracelular conlleva la diseminación intraorgánica del parásito y el consiguiente daño tisular (Dubey *et al.*, 2006). Los taquizoítos son capaces de invadir una amplia variedad de tipos celulares tales como neuronas, macrófagos, células dendríticas, fibroblastos, células endoteliales, miocitos y hepatocitos, por lo que su diseminación intraorgánica es rápida y efectiva (Collantes-Fernández *et al.*, 2012; Dubey *et al.*, 2002a; Barr *et al.*, 1993). Por su parte, los bradizoítos participan en la fase crónica de la infección. Éstos presentan una tasa de replicación más baja y se localizan en el interior de quistes tisulares formados en el espacio intracitoplasmático de una sola célula, predominantemente en el tejido nervioso (Barr *et al.*, 1991b). Los esporozoítos constituyen la forma de transmisión ambiental, y se forman a partir de los ooquistes, que son eliminados al medio ambiente por el hospedador definitivo. Bajo condiciones ambientales favorables, los ooquistes esporulan y forman en su interior dos esporoquistes con cuatro esporozoítos cada uno, pudiendo contener un cuerpo residual (Lindsay *et al.*, 1999d; McAllister *et al.*, 1998). A pesar de que se ha demostrado la presencia de estadios enteroepiteliales del parásito en perros naturalmente infectados (Kul *et al.*, 2015), los estadios de merozoíto y gametocito, ampliamente estudiados en *T. gondii*, no han sido bien caracterizados en *N. caninum* (Hehl *et al.*, 2015; Dubey *et al.*, 1998a).

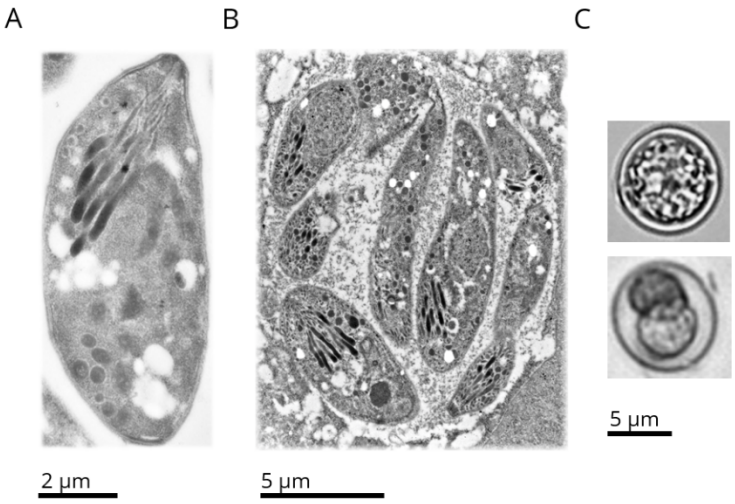


Figura 1. Imágenes de microscopía electrónica de un taquizoíto (A) y un quiste con bradizoítos (B) obtenidos *in vitro*. Imágenes de microscopía óptica de un ooquiste de la familia Toxoplasmatinae sin esporular y otro esporulado (C).

Tanto los taquizoítos como los bradizoítos y los esporozoítos presentan las organelas típicas de cualquier célula ecuariota (núcleo, retículo endoplásmico, aparato de Golgi, mitocondria, etc.), así como

otras estructuras específicas de los parásitos apicomplejos (Dubey *et al.*, 2002a) (Figura 2). Los Apicomplexa presentan externamente una película semi-rígida formada por tres bicapas. La membrana plasmática constituye la bicapa más externa, mientras que el complejo interno de la membrana constituye las dos restantes. Este complejo está formado por alveolos aplanados que interaccionan externamente con el glideosoma e internamente con los microtúbulos subpeliculares (Ouologuem & Roos, 2014). El glideosoma agrupa a los elementos que componen el motor de actomiosina del parásito, que genera la fuerza mecánica necesaria para invadir a la célula hospedadora (Boucher & Bosch, 2015). Los microtúbulos subpeliculares ejercen la función de citoesqueleto, puesto que recorren longitudinalmente a los zoítos. Estas estructuras se anclan en dos anillos polares anteriores, que albergan el conoide, y un anillo polar posterior. Dicho conoide es un cono truncado y móvil constituido por filamentos de tubulina (Speer *et al.*, 1999; Lindsay *et al.*, 1993; Speer & Dubey, 1989).

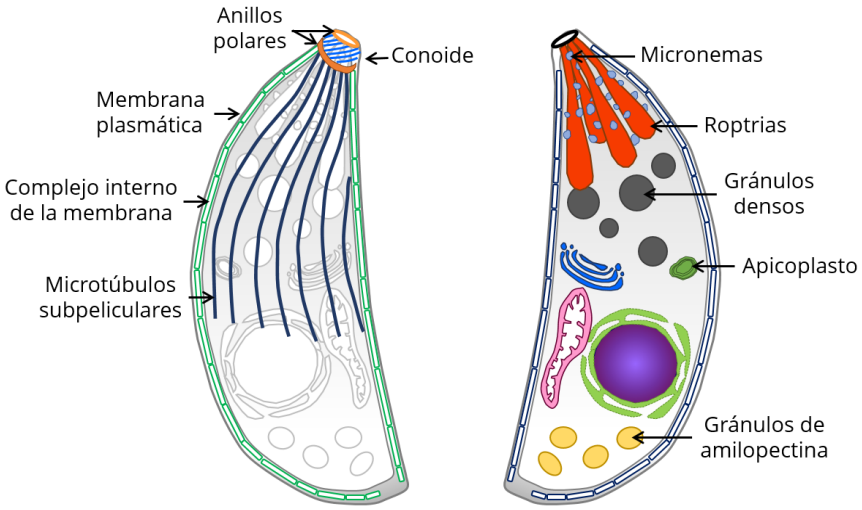


Figura 2. Representación gráfica de la ultraestructura del taquizoíto de *N. caninum*, con especial mención a los componentes del citoesqueleto (izquierda) y del citoplasma (derecha) únicamente encontrados en protozoos apicomplejos.

El citoplasma de los zoítos presenta cuatro organelas de secreción denominadas roptrias, micronemas, gránulos densos y gránulos de amilopectina, cuyo número y distribución tiene valor taxonómico por ser específico de cada género, especie y estadio parasitario (Dubey *et al.*, 2002a). Mientras que las roptrias y los micronemas se localizan en el polo anterior del parásito y confluyen en el conoide, los gránulos densos y de amilopectina se distribuyen homogéneamente en citoplasma del mismo (Figura 2). En concreto, el término de complejo apical hace referencia al conjunto de conoide, anillos polares, micronemas y roptrias (Dubey *et al.*, 1988). Además, cabe destacar la presencia del apicoplasto, que es una organela vestigial con estructura similar a la de los plástidos de las algas, aunque sin capacidad fotosintética. El apicoplasto está presente en la mayoría de los Apicomplexa, aunque no existe en *Cryptosporidum parvum* (Zhu *et al.*, 2000). Su presencia es esencial para el resto de los géneros, por lo que

se presenta como una importante diana terapéutica. Esta organela contiene su propio material genético, que codifica elementos esenciales para el metabolismo del parásito (síntesis de ácidos grasos, isoprenoides y grupos hemo), e importa ciertas proteínas procedentes del núcleo (Sheiner *et al.*, 2013).

1.2. Ciclo biológico y transmisión

El ciclo biológico de *N. caninum* es heteroxeno facultativo (Figura 3). Hasta el momento se han confirmado al perro, coyote, dingo y lobo como hospedadores definitivos (Dubey *et al.*, 2011; King *et al.*, 2010; Gondim *et al.*, 2004), aunque también pueden actuar como hospedadores intermediarios. En ellos se produciría la reproducción sexual del parásito con la consiguiente formación de ooquistes tras la esquizogonia y la gametogonia (McAllister *et al.*, 1998). No obstante, estas fases alternantes de reproducción asexual y sexual sólo han sido descritas en *T. gondii* (Elmore *et al.*, 2010), aunque se asumen similares en *N. caninum* (Kul *et al.*, 2015; Dubey *et al.*, 2004).

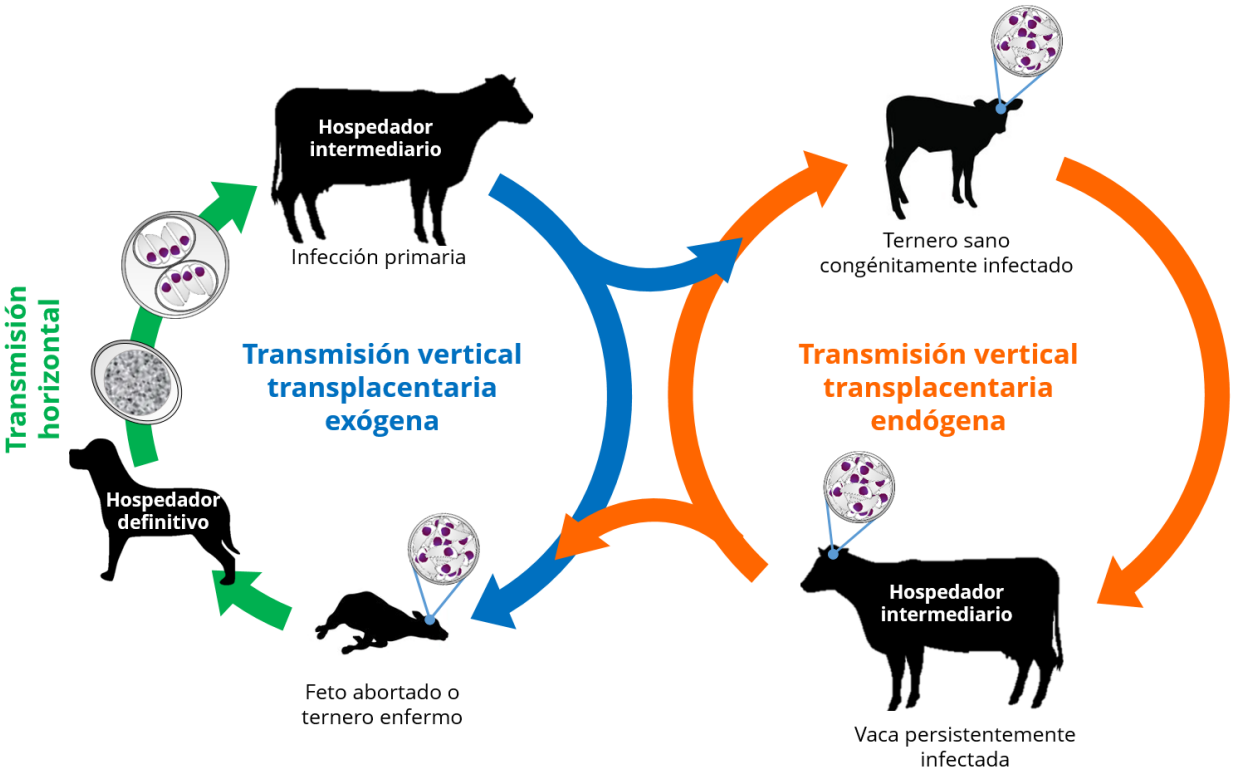


Figura 3. Ciclo biológico y modos de transmisión de *N. caninum*.

Los hospedadores definitivos liberan con las heces ooquistes que esporulan tras un mínimo de 24 horas en el medio ambiente bajo condiciones favorables (Lindsay *et al.*, 1999a). Como en otros coccidios, los ooquistes tienen una resistencia ambiental muy elevada (Alves Neto *et al.*, 2011; Uzeda *et al.*, 2007). Sin embargo, parece ser que los perros infectados de forma natural eliminan una baja cantidad de ooquistes, que además se reduce con la edad (Gondim *et al.*, 2005; Schares *et al.*, 2005). Por otro lado, la infección del perro mediante la ingestión de ooquistes esporulados sólo parece producirse a altas dosis y, en cualquier caso, no va asociada a una liberación posterior de ooquistes con las heces, por lo que en

estos casos actuaría como hospedador intermediario (Bandini *et al.*, 2011). No obstante, el hospedador intermediario principal es el ganado vacuno, aunque la exposición de una gran variedad de animales domésticos y silvestres a *N. caninum* ha sido puesta de manifiesto en diversos estudios. Hasta la fecha se ha aislado taquizoítos viables de la vaca, la oveja, el búfalo de agua, el perro, el lobo, el caballo, el bisonte y el ciervo de cola blanca. Sin embargo, se ha demostrado la presencia de ADN del parásito o de una respuesta serológica frente al mismo en otras especies (zorro rojo, mapache, antílope, ciervo de cola blanca, ciervo de Eld, gamo, llama, alpaca, cabra, rinoceronte, rata, ratón, rata topera, topillo, capibara, conejo, liebre, gallina, gorrión, cuervo, ratonero y urraca) (Dubey *et al.*, 2014; Darwich *et al.*, 2012; Dubey & Schares, 2011). Por otro lado, y pese a su gran homología con *T. gondii* (Reid *et al.*, 2012), no existen evidencias de que *N. caninum* pueda infectar al hombre (McCann *et al.*, 2008; Dubey *et al.*, 2007a).

El ganado bovino puede adquirir la infección por *N. caninum* mediante dos modos de transmisión: horizontal y vertical (Figura 3). La ingestión de ooquistes esporulados es el único modo de transmisión horizontal demostrado hasta el momento (Dubey *et al.*, 2007a), puesto que la transmisión venérea parece no ocurrir en condiciones naturales (Serrano *et al.*, 2006). Una vez ingeridos, los esporozoítos infectan a las células del epitelio intestinal, donde se convierten en taquizoítos de rápida replicación y alcanzan el torrente circulatorio a través de los linfonódulos mesentéricos (Dubey *et al.*, 2006; Lindsay *et al.*, 1999b). La transmisión vertical de la infección puede ser lactogénica o transplacentaria. La transmisión lactogénica sólo ha sido demostrada experimentalmente, y parece poco probable en condiciones naturales (Moskwa & Cabaj, 2007; Davison *et al.*, 2001; Dijkstra *et al.*, 2001). Por el contrario, la transmisión transplacentaria es muy efectiva en el ganado bovino, y probablemente en otros hospedadores infectados. De hecho, hasta un 95% de los terneros nacidos de madres infectadas presentan la infección (Dubey *et al.*, 2007a). Este tipo de transmisión tiene tres posibles consecuencias, que se asocian a la replicación y diseminación de los taquizoítos: el aborto, el nacimiento de terneros con signos clínicos y congénitamente infectados y el nacimiento de terneros normales y congénitamente infectados (Williams *et al.*, 2009).

Desde el punto de vista del origen de la infección, se han descrito dos modos de transmisión transplacentaria: la exógena y la endógena. Ambos modos presentan consecuencias patogénicas diferentes, por lo que sus medidas de control también lo son. La transmisión transplacentaria exógena se presenta en vacas primo-infectadas durante la gestación por la ingestión de ooquistes esporulados del medio ambiente. Por el contrario, la transmisión transplacentaria endógena aparece en vacas crónicamente infectadas portadoras de quistes con bradizoítos, que sufrirán una reactivación de la infección durante la gestación (Trees & Williams, 2005). Este último modo de transmisión parece ser el responsable de mantener la infección en el rebaño, puesto que las hembras crónicamente infectadas son capaces de transmitir el parásito a su descendencia en gestaciones sucesivas (Hall *et al.*, 2005; Piergili *et al.*, 2003).

Independientemente del modo de transmisión, el hospedador es capaz de desarrollar una respuesta inmunitaria que controla la replicación y diseminación de los taquizoítos. Una vez instaurada, el parásito evade dicha respuesta mediante su conversión al estadio de bradizoíto. Los bradizoítos se disponen en el interior de quistes tisulares que se localizan en el tejido nervioso y en la musculatura estriada, donde reducen su tasa de replicación. Esto permite la cronificación de la infección en el tiempo y la evasión de la respuesta inmunitaria del hospedador (Buxton *et al.*, 2002; Peters *et al.*, 2001). De tal modo, la depredación de tejidos infectados con quistes de bradizoítos constituye el principal modo de transmisión de la infección desde el hospedador intermediario al definitivo, mientras que la reactivación de dichos bradizoítos durante la gestación constituye el principal modo de transmisión entre dos hospedadores intermediarios (Dubey & Schares, 2011).

1.3. Signos clínicos

La neosporosis bovina es una enfermedad que afecta principalmente a la placenta y al feto, por lo que sus manifestaciones clínicas se restringen a la presentación de aborto y, en menor medida, al nacimiento de terneros con signos clínicos. Los fetos pueden morir en el útero o ser reabsorbidos, momificados, sufrir autolisis, nacer muertos, nacer vivos con signos clínicos neuromusculares o nacer clínicamente normales, pero persistentemente infectados (Benavides *et al.*, 2014; Dubey *et al.*, 2007a).

Las hembras infectadas de forma natural pueden presentar aborto durante todo el año de forma esporádica (menos del 5% de abortos al año), endémica (entre un 10 y un 12,5% de abortos a lo largo varios meses o un año) y epidémica (más de un 12,5% de abortos en menos de 6-8 semanas) (Dijkstra *et al.*, 2001; Wouda *et al.*, 1999; Thurmond & Hietala, 1997b). Los abortos epidémicos se presentan en forma de tormentas y se han asociado a una primo-infección tras el contacto con ooquistes esporulados, con la consiguiente transmisión transplacentaria exógena. Por el contrario, los abortos endémicos son característicos de explotaciones crónicamente infectadas donde se producen reactivaciones de la infección que desembocan en una transmisión transplacentaria endógena (Dubey *et al.*, 2007a; Pabon *et al.*, 2007; Trees & Williams, 2005). En ambos casos, las vacas afectadas mantienen la infección de por vida y pueden transmitirla en gestaciones sucesivas o intermitentes (Fioretti *et al.*, 2003; Guy *et al.*, 2001; Wouda *et al.*, 1998; Boulton *et al.*, 1995).

Aunque es poco frecuente, en determinadas ocasiones los terneros pueden nacer muy débiles y con signos neurológicos, por lo que acaban muriendo durante el primer mes de vida (Dubey *et al.*, 2006). Estos signos incluyen incoordinación y parálisis, que suelen ir asociados a la aparición de diversas malformaciones congénitas como la hidrocefalia o el estrechamiento de la médula espinal (Bryan *et al.*, 1994; Gunning *et al.*, 1994; Dubey *et al.*, 1992).

1.4. Patogenia y lesiones

La presentación del aborto por *N. caninum* está influenciada por numerosos factores dependientes del parásito (aislado, estadio, vía de inoculación y dosis infectante) y del hospedador (inmunidad materna e inmunocompetencia fetal, edad gestacional y origen de la infección). No obstante, la mayoría de la información de la que se dispone en la actualidad se ha obtenido a través de infecciones experimentales, por lo que el conocimiento de la patogenia de la enfermedad en condiciones naturales es limitado (Benavides *et al.*, 2014).

En primer lugar, las vacas infectadas de forma exógena presentan tasas de transmisión vertical menores que aquellas que sufren reactivaciones endógenas, y en algunos casos son capaces de eliminar la infección (Dijkstra *et al.*, 2008). Del mismo modo, las tasas de transmisión vertical parecen disminuir con la edad de la hembra, probablemente debido al desarrollo de inmunidad frente al parásito, lo que produciría una eliminación progresiva de la infección del rebaño afectado hasta un punto en el que ésta desapareciera (Dijkstra *et al.*, 2003; Romero *et al.*, 2002). No obstante, los modos de transmisión transplacentaria endógena y exógena parecen complementarse para impedir dicha eliminación. Así lo demuestra la presentación de abortos epidémicos, los incrementos de seropositividad de las hembras con la edad, la aparición de hijas seropositivas de madres seronegativas y la elevada seroprevalencia de los perros de granja (Collantes-Fernández *et al.*, 2008; Huang *et al.*, 2004; Dyer *et al.*, 2000). De hecho, en España se han descrito tres escenarios epidemiológicos distintos en los que la transmisión transplacentaria exógena y endógena pueden presentarse de forma independiente o combinada (Rojo-Montejo *et al.*, 2009c).

En segundo lugar, los abortos producidos por *N. caninum* son más frecuentes cuando la infección se produce durante el primer y segundo tercio de la gestación (Williams *et al.*, 2009; Buxton *et al.*, 2002), mientras que la infección en el último tercio da lugar al nacimiento de terneros congénitamente infectados sanos o con signos clínicos (Reichel *et al.*, 2014). A día de hoy, las bases patogénicas que desencadenan el aborto no han sido descifradas por completo, aunque se encuentran íntimamente ligadas a la diseminación intraorgánica y daño tisular producido por los taquizoítos. Hasta el momento se han postulado cuatro mecanismos por los que se podría producir el aborto: la multiplicación de los taquizoítos en los órganos vitales del feto, el daño de la placenta por el parásito, con la consecuente alteración del suministro de oxígeno y nutrientes al feto, la alteración del balance inmunitario a nivel de la placenta, y la secreción de prostaglandinas que inducen el aborto y el daño fetal (Dubey *et al.*, 2007a; Innes, 2007; Dubey *et al.*, 2006; Innes *et al.*, 2005; Buxton *et al.*, 2002). Recientemente, se ha descrito un nuevo mecanismo de aborto en el modelo ovino de toxoplasmosis aguda. Dicho mecanismo está basado en la aparición de áreas de infarto y trombosis a nivel de los placentomas, donde no existe evidencia alguna de multiplicación del parásito (Castaño *et al.*, 2014). Sin embargo, se desconoce la posibilidad de que *N. caninum* pueda desencadenar este mismo fenómeno.

En tercer lugar, la respuesta inmunitaria materna y fetal son factores fundamentales durante el transcurso de la infección, ya que tienen un papel clave en la presentación o no del aborto. En condiciones naturales, el periodo gestacional se acompaña de una regulación fisiológica hacia una respuesta inmunitaria Th2 (Entrican, 2002). Ésta evita el rechazo del feto, pero favorece la diseminación y multiplicación del parásito en los tejidos de la madre y el feto. Por el contrario, el desarrollo de una respuesta celular Th1 ha demostrado ser eficaz frente al parásito, pero podría desencadenar el rechazo del feto. Por lo tanto, parece esencial que se produzca un equilibrio Th1/Th2 que permita el mantenimiento de la gestación sin que se produzcan alteraciones significativamente graves para el feto (Innes *et al.*, 2002). En general, la infección durante el primer tercio de la gestación se traduce en aborto asociado al desarrollo de respuestas inflamatorias en la placenta (Canton *et al.*, 2014; Rosbottom *et al.*, 2008; Maley *et al.*, 2006) y a la falta de desarrollo inmunológico del feto, donde el parásito se multiplica sin control (Innes *et al.*, 2001b; Williams *et al.*, 2000). Dado que los órganos linfoides fetales inician su maduración a partir del segundo tercio de la gestación, a partir de dicho periodo los fetos son capaces de controlar en cierto grado a la infección, aunque nacen congénitamente infectados (Williams *et al.*, 2009; Quintanilla-Gozalo *et al.*, 2000). Por otro lado, la co-infección con BVD y la ingesta de micotoxinas se ha relacionado con una mayor susceptibilidad al aborto por *N. caninum*, probablemente debida a una inmunosupresión secundaria que supondría una alteración del balance Th1/Th2 (Björkman *et al.*, 2000; Bartels *et al.*, 1999).

Por último, la diversidad biológica de *N. caninum* tiene una clara influencia en la patogenia de la enfermedad. De hecho, los aislados estudiados hasta el momento presentan diferencias marcadas en su virulencia que determinan la presentación o no del aborto, al menos experimentalmente. Esta diversidad será abordada a fondo en epígrafes posteriores (*4. Variabilidad intra-específica de* N. caninum). Por otro lado, se ha determinado de forma experimental que la inoculación intravenosa de taquizoítos conlleva consecuencias más graves para el feto que la subcutánea y que la infección oral con ooquistes esporulados (Rojo-Montejo *et al.*, 2009a; McCann *et al.*, 2007; Macaldowie *et al.*, 2004). Del mismo modo, la administración de mayores dosis del parásito va asociada a mayores tasas de mortalidad neonatal y al desarrollo de lesiones tisulares más graves, al menos en los modelos murino y ovino (Arranz-Solís *et al.*, 2015a; Arranz-Solís *et al.*, 2015b; Collantes-Fernández *et al.*, 2004; Long *et al.*, 1998).

La replicación de los taquizoítos en los tejidos del hospedador conlleva el desarrollo de ciertas lesiones microscópicas que, aunque no son patognomónicas, facilitan el diagnóstico del aborto. Las lesiones se focalizan principalmente en la placenta y el feto, siendo siempre de tipología inflamatoria y no supurativa. Por lo general, la gravedad de las lesiones, manifestada en forma de necrosis y calcificaciones, es mayor cuando la infección y el aborto se producen más tempranamente (Gibney *et al.*, 2008; Collantes-Fernández *et al.*, 2006a; Collantes-Fernández *et al.*, 2006c). En los fetos con infección congénita las lesiones se concentran en el sistema nervioso central, con áreas multifocales de infiltración linfocitaria, manguitos

perivasculares, microgliosis, presencia de astrocitos y áreas de necrosis. También se pueden observar focos de inflamación no supurativa en el corazón y el hígado, y en menor medida en los riñones, musculatura esquelética y pulmón. Curiosamente, la gravedad de estas lesiones es mayor cuando el aborto es debido a una transmisión transplacentaria exógena (Dubey *et al.*, 2006). Los terneros congénitamente infectados que nacen con signos nerviosos presentan predominantemente lesiones inflamatorias a nivel espinal y, menos frecuentemente, en el encéfalo (Peters *et al.*, 2001). Por el contrario, la presencia de lesiones en los terneros congénitamente infectados sin signos clínicos y en animales adultos es rara y se restringe al sistema nervioso central (Sawada *et al.*, 2000; Bryan *et al.*, 1994; Barr *et al.*, 1991a).

1.5. Impacto de la neosporosis bovina

Los estudios en los que se ha analizado la prevalencia de la infección por *N. caninum* destacan diferencias importantes entre países y regiones, y entre los diferentes sistemas de producción. No obstante, los datos registrados son difíciles de comparar porque han sido obtenidos mediante técnicas de diagnóstico diferentes, con diseños experimentales y tamaños de muestra muy variables (Dubey & Schares, 2011). No obstante, en un estudio llevado a cabo en Alemania, España, Holanda y Suecia bajo técnicas serológicas estandarizadas y diseños experimentales similares mostró grandes diferencias en las seroprevalencias individuales de cada país, siendo muy baja en Suecia y Alemania, y de moderada a alta en Holanda y España (Bartels *et al.*, 2006). Por otro lado, se han observado seroprevalencias más elevadas en perros procedentes de granjas que en perros procedentes de núcleos urbanos (Dubey & Schares, 2011; Regidor-Cerrillo *et al.*, 2010b).

En el noroeste de España las seroprevalencias de rebaño alcanzan el 79,3 - 87,7% en ganado de aptitud láctea, y el 76,7% en ganado de aptitud cárnica. A nivel individual, las seroprevalencias oscilan entre el 15,7 y el 21,9% en los rebaños de aptitud láctea, y el 25,1% en los de aptitud cárnica (Eiras *et al.*, 2011; Gonzalez-Warleta *et al.*, 2008). Además, se ha descrito un incremento de la seroprevalencia con la edad de los animales asociado a un contacto con el parásito por el modo de transmisión horizontal a lo largo de su vida (Eiras *et al.*, 2011). La tasa de infección de fetos abortados varía entre el 22 y el 58%, señalando a *N. caninum* como un importante agente abortivo dentro de la geografía española, seguido distancia por el virus de la diarrea vírica bovina (BDV) (Pereira-Bueno *et al.*, 2003; Aduriz *et al.*, 2001; Aduriz *et al.*, 1999; Gonzalez *et al.*, 1999).

La neosporosis bovina tiene un impacto económico negativo en la producción ganadera. Éste deriva de la aparición de abortos y de otros efectos indirectos, tales como el incremento del intervalo entre partos, la reducción de la producción láctea y de la tasa de crecimiento, la pérdida del valor genético de los animales por su eliminación temprana y el incremento de los costes veterinarios (Barling *et al.*, 2001; Barling *et al.*, 2000; Trees *et al.*, 1999; Thurmond & Hietala, 1997a; Thurmond & Hietala, 1996). Las pérdidas

económicas parecen ser más importantes en la industria del ganado de leche, donde el riesgo de aborto es mayor (Moore *et al.*, 2009). Recientemente, el impacto de la enfermedad ha sido estimado en un billón de dólares, si bien este estudio se restringe a las principales potencias ganaderas a nivel mundial, por lo que se cree que puede ser notablemente superior (Reichel *et al.*, 2013). Los factores de riesgo de la neosporosis bovina son numerosos y han sido exhaustivamente revisados en los últimos años (Dubey *et al.*, 2007a). Entre otros, destaca la presencia de perros en las granjas y el acceso de los mismos a los fetos abortados y placentas, el propio manejo del rebaño (método de cría, gestión de pastos y alojamientos, densidad de animales), la humedad y pluviometría del área geográfica, y la co-existencia de infecciones por los virus de del BDV e IBR (Dubey & Schares, 2011).

1.6. Diagnóstico

El diagnóstico de la neosporosis bovina no es fácil y debe abordarse de forma adecuada y sistemática. Puesto que los animales mantienen la infección de por vida, la detección de anticuerpos específicos frente a *N. caninum* ha demostrado ser un método de diagnóstico fiable. No obstante, la seropositividad de un animal no implica necesariamente la presentación de abortos en el futuro. Si bien existen evidencias de que las mayores seroprevalencias individuales y de rebaño están directamente relacionadas con una mayor incidencia del aborto, otros factores como la edad se han asociado a un descenso de la incidencia del mismo (Dubey *et al.*, 2007a). Por todo ello, la asociación de un aborto a la presencia de *N. caninum* sólo es posible tras un estudio de la respuesta serológica de la madre y la detección del parásito y sus lesiones asociadas en el feto abortado (Álvarez-García *et al.*, 2007; Dubey & Schares, 2006).

Hasta la fecha se han desarrollado diferentes técnicas de diagnóstico serológico que permiten realizar el diagnóstico post-natal de la neosporosis bovina. La prueba de ELISA es la más utilizada y es aplicable en animales de más de 6 meses y en recién nacidos, siempre y cuando se realice sobre sueros precalostrales. La utilidad del diagnóstico serológico es mayor cuando este se plantea a nivel de rebaño, puesto que permite monitorizar el progreso de la infección y establecer medidas de control adecuadas para su situación epidemiológica concreta (Álvarez-García *et al.*, 2013b). Concretamente, los ELISAs de avidez son capaces de diferenciar infecciones recientes de crónicas (Björkman *et al.*, 2006; Schares *et al.*, 2002), mientras que los ELISAs basados en las proteínas NcGRA7 y NcSAG4 permiten diferenciar entre primo-infección, recrudescencia e infección crónica (Aguado-Martínez *et al.*, 2008).

Por el contrario, el valor de la serología como herramienta diagnóstica del aborto es muy limitado. La respuesta serológica de un feto frente a la infección por *N. caninum* depende del grado de desarrollo de su sistema inmunitario en el momento del aborto y, por lo tanto, del momento de la gestación en el que adquirió la infección (Wouda *et al.*, 1997). Por esta razón, las técnicas de elección para el diagnóstico del aborto se basan en la detección del parásito y las lesiones que provoca mediante PCR,

inmunohistoquímica e histología. En este sentido, los órganos de elección son siempre el encéfalo y la placenta (Álvarez-García *et al.*, 2007; Buxton *et al.*, 1998).

1.7. Control

En la actualidad sólo se dispone de medidas de manejo para luchar contra la neosporosis bovina, y su establecimiento dependerá de la situación epidemiológica de cada granja (seroprevalencia y modo de transmisión) y de la relación coste-beneficio. El refuerzo de la bioseguridad de las granjas libres de la infección es clave para que éstas puedan mantener su estatus sanitario. Esto incluiría la cuarentena y el estudio serológico de los animales procedentes de otras explotaciones, el control del acceso de los perros a las zonas de almacenamiento de comida, comederos y bebederos, la eliminación segura de restos de abortos y el control de otros reservorios. Análogamente, estas rutinas deberían implementarse en aquellas granjas que hubieran sufrido un brote epidémico de abortos por una primo-infección y la consecuente transmisión transplacentaria exógena. En las explotaciones donde la infección está presente de forma endémica las medidas de control pretenden frenar la transmisión transplacentaria endógena y reducir la diseminación del parásito a la descendencia. Para ello, se recomienda realizar sacrificios selectivos de forma paulatina, empezando por las vacas que abortan y continuando con las vacas seropositivas. No obstante, estas medidas son sólo viables en granjas con prevalencias muy bajas. Cuando las tasas de prevalencia son tan elevadas que el sacrificio selectivo resulta económicamente inviable, se puede plantear una eliminación progresiva de la infección mediante reposición selectiva con hijas de vacas seronegativas y destinar a las crías de las vacas seropositivas a la producción de carne. En el caso de animales seropositivos de alto valor genético, se puede recurrir a la transferencia de embriones usando vacas seronegativas como receptoras, o bien, realizar inseminación artificial con razas cárnicas, lo cual se ha asociado a una reducción de la probabilidad de aborto (Reichel *et al.*, 2013; Dubey *et al.*, 2007a). El seguimiento serológico a nivel de rebaño sirve como un indicador de la eficacia de estos programas de control, que en ningún caso deben obviar las medidas de bioseguridad mencionadas previamente. En este sentido, cabe destacar la eficacia de algunos programas de control frente a la enfermedad llevados a cabo en Holanda, España, Portugal y Bélgica, y que se han fundamentado en el estudio serológico tanto a nivel individual, mediante muestras de sangre, como a nivel de rebaño, mediante análisis de tanque de leche. En general, estos programas se han centrado en la eliminación progresiva de los animales seropositivos con problemas de abortos, así como en una reposición selectiva con animales seronegativos. En el caso concreto de Holanda, la prevalencia de rebaño de la enfermedad descendió casi un 14% en sólo 3 años, mientras que este descenso fue del 3-7% en España (Reichel *et al.*, 2014).

El uso de fármacos no parece una aproximación viable para luchar frente a la neosporosis bovina, ya que éstos sólo serían eficaces durante la fase aguda de la infección, en la que los signos clínicos pasan prácticamente inadvertidos. Hasta el momento el toltrazuril ha sido el único fármaco que ha demostrado ser eficaz frente a las infecciones experimentales llevadas a cabo en terneros (Kritzner *et al.*, 2002). Sin

embargo, se duda de su utilidad práctica en infecciones naturales y tiene un elevado coste. Por otro lado, y aunque su eficacia sólo se ha comprobado *in vitro* y/o en ratones, diversos compuestos frente a *N. caninum* han sido testados con resultados variables (Müller *et al.*, 2015a; Müller *et al.*, 2015b; Ojo *et al.*, 2014; Barna *et al.*, 2013; Seo *et al.*, 2013; Debache & Hemphill, 2012; Mazuz *et al.*, 2012).

En un reciente análisis coste-beneficio, la vacunación frente a la neosporosis bovina ha demostrado ser la medida de control más rentable desde el punto de vista económico (Reichel *et al.*, 2013). Durante los últimos años, los esfuerzos de diferentes grupos de investigación se han dirigido al desarrollo de vacunas vivas por ser las que mejor inducirían una respuesta inmunitaria en el hospedador. Sin embargo, y pese a los buenos resultados en términos de eficacia asociados a su empleo (Rojo-Montejo *et al.*, 2013), aún no existen vacunas vivas comerciales debido a la dificultad de su producción a gran escala (Reichel *et al.*, 2015). Por esta razón, las vacunas inactivadas y de subunidades han emergido como interesantes alternativas de las vacunas vivas (Monney & Hemphill, 2014).

A día de hoy el control de la neosporosis bovina depende exclusivamente de un manejo adecuado y apoyado en el diagnóstico de los animales. No obstante, es obvio que existe una necesidad urgente de desarrollar medidas de control que prevengan o eliminen la infección, y que sean viables desde el punto de vista económico. En vistas a los problemas de estabilidad y seguridad asociados al uso de vacunas vivas, parece fundamental ahondar en los mecanismos de virulencia de *N. caninum*, prácticamente desconocidos hasta la fecha, y determinar las dianas más aptas para el desarrollo de vacunas de subunidades contra la enfermedad.

2. La interacción del parásito con la célula hospedadora: hacia la búsqueda de nuevas dianas vacunales

Como consecuencia de su elevada adaptación a los hospedadores a los que parasita, *N. caninum* ha desarrollado diversas estrategias que garantizan su supervivencia y multiplicación en un ambiente estrictamente intracelular. Esto implica que durante los procesos clave del ciclo biológico, como son la infección inicial durante la fase aguda y la conversión a bradizoíto durante la fase crónica de la infección, la interacción del parásito con las células hospedadoras se encuentre íntimamente regulada (Dubey *et al.*, 2007a). En este sentido, los componentes involucrados en ambos procesos podrían ser candidatos vacunales ideales frente a la enfermedad, puesto que su bloqueo permitiría alterar procesos esenciales para la supervivencia del parásito. Pese a ello, a día de hoy los mecanismos de interacción entre *N. caninum* y sus células hospedadoras son bastante desconocidos.

2.1. Dinámica de la infección: taquizoítos y bradizoítos

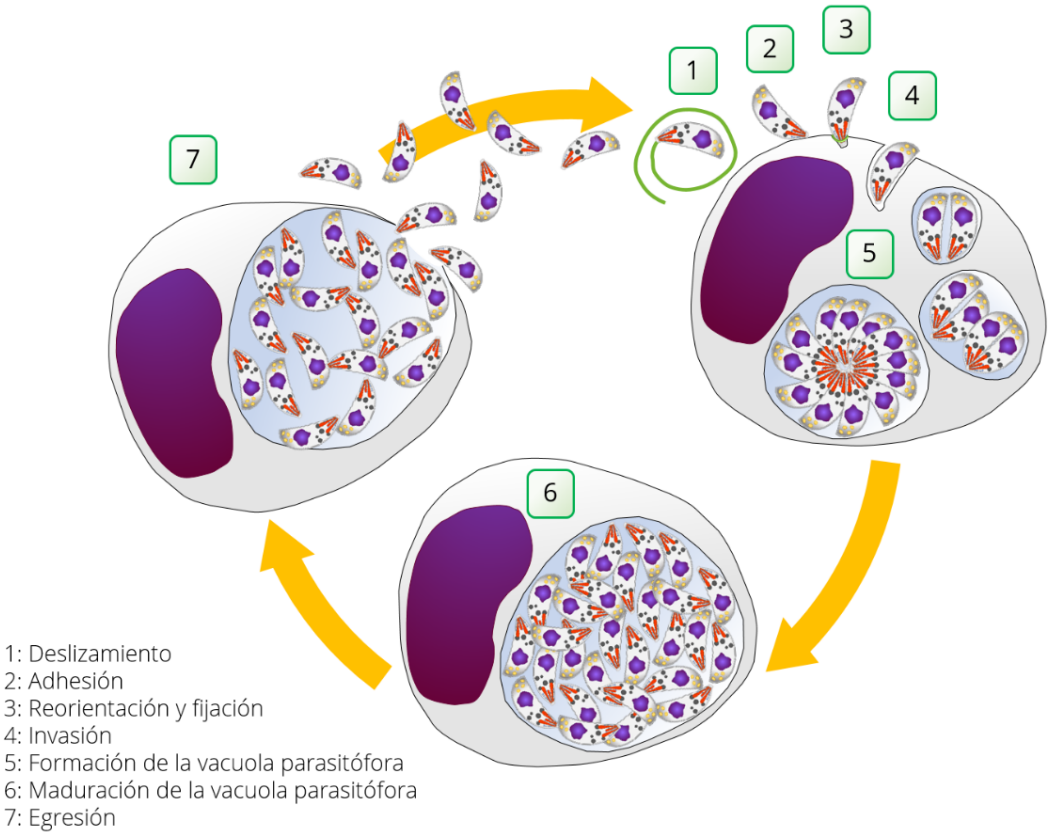
La infección por *N. caninum* en el hospedador intermediario es un proceso dinámico y controlado en el que participan de forma coordinada dos estadios parasitarios: los taquizoítos, responsables de la fase aguda de la infección, y los bradizoítos, responsables de la fase crónica de la misma. Los taquizoítos se caracterizan por su elevada tasa de replicación por endodiogenia y por su gran capacidad para infectar diversos tipos celulares, lo que les permite diseminarse por todo el organismo. Por su parte, los bradizoítos se acantonan dentro de quistes localizados en órganos inmunoprivilegiados, donde reducen su velocidad de replicación y su exposición al sistema inmune (Dubey *et al.*, 2006).

Tras la invasión, los taquizoítos originan una vacuola parasitófora dentro de las células infectadas que les permite multiplicarse activamente. Dicha vacuola se genera a partir de la membrana plasmática de la propia célula hospedadora y, posteriormente, es modificada por parte del parásito para impedir su fusión con los lisosomas y su consiguiente destrucción (Mordue *et al.*, 1999a; Mordue *et al.*, 1999b; Hemphill *et al.*, 1996). Además, la vacuola parasitófora es capaz de reclutar a las organelas de la célula hospedadora y de secuestrar su maquinaria metabólica en favor de la replicación del parásito (Nolan *et al.*, 2015). En su interior, los taquizoítos experimentan ciclos sucesivos de replicación asexual hasta desencadenarse la egresión del parásito al medio extracelular, lo que origina la lisis de la célula hospedadora y la posterior invasión de las células vecinas por los zoítos liberados, iniciándose un nuevo ciclo de proliferación (ciclo lítico) (Hemphill, 1999).

En el transcurso de una infección aguda, el sistema inmunitario del hospedador es capaz de desarrollar una respuesta específica frente a los taquizoítos circulantes. La mayoría de ellos son destruidos de forma efectiva, aunque aquellos presentes en órganos inmunoprivilegiados son capaces de diferenciarse intracelularmente a bradizoítos y enquistarse (Dubey *et al.*, 2006). La formación de quistes tisulares da lugar la cronificación de la enfermedad, haciendo que el hospedador mantenga la infección de por vida (Dubey & Lindsay, 1996). En *T. gondii* la conversión al estadio de bradizoíto parece comenzar tan sólo un día después de haberse producido la infección (Di Cristina *et al.*, 2008), aunque las causas que la originan no son del todo conocidas. Aparentemente, la formación de bradizoítos depende de diversos factores exógenos, como son los dependientes de la célula hospedadora, y otros factores endógenos que afectan directamente el parásito. Curiosamente, las diferentes líneas clonales de *T. gondii* difieren en su capacidad para iniciar la latencia de la infección. No obstante, se desconocen las bases genéticas que determinan estas diferencias. El proceso de conversión a bradizoíto parece surgir como una respuesta a diversos agentes estresantes, que acaban por modificar el perfil transcripcional del parásito y desatan importantes cambios. Entre ellos destacan la polarización hacia rutas anaerobias del metabolismo de los carbohidratos, el estancamiento en la fase G0 del ciclo celular y la modificación de las cascadas de señalización asociadas al proceso de egresión (Sullivan & Jeffers, 2012).

2.2. Ciclo lítico de los taquizoítos

El ciclo lítico es un proceso altamente conservado entre todos los parásitos apicomplejos y que incluye las fases de deslizamiento, adhesión, reorientación, fijación, invasión, establecimiento de la vacuola parasitófora, proliferación y egresión (Figura 4). En su conjunto, permiten que el taquizoíto pueda multiplicarse de forma masiva y diseminarse por diversos tejidos durante la fase aguda de la infección. Al ser un proceso clave para la supervivencia del parásito, la progresión de sus distintas etapas se encuentra estrechamente regulada. En concreto, las proteínas de la superficie del taquizoíto (SRS), así como aquellas secretadas desde los micronemas (MIC), el cuello y cuerpo de las roptrias (RON y ROP) y los gránulos densos (GRA), actúan coordinadamente sobre la célula hospedadora y permiten el avance secuencial del ciclo. Las bases moleculares del ciclo lítico han sido estudiadas en detalle en *T. gondii* (Carruthers & Boothroyd, 2007) y más superficialmente en *N. caninum* (Hemphill *et al.*, 2004; Hemphill *et al.*, 1996). No obstante, dada la elevada homología que presentan las proteínas SRS, MIC, RON, ROP y GRA de *T. gondii* y *N. caninum*, se asume que ambos organismos comparten mecanismos de regulación similares (Hemphill *et al.*, 2013). Pese a ello, y teniendo en cuenta las diferencias que presentan en su espectro de hospedadores y en sus modos de transmisión de la infección, se cree que ambos protozoos presentan variaciones notables en la interacción con sus respectivas células hospedadoras (Reid *et al.*, 2012; Innes & Mattsson, 2007; Hemphill *et al.*, 2006).



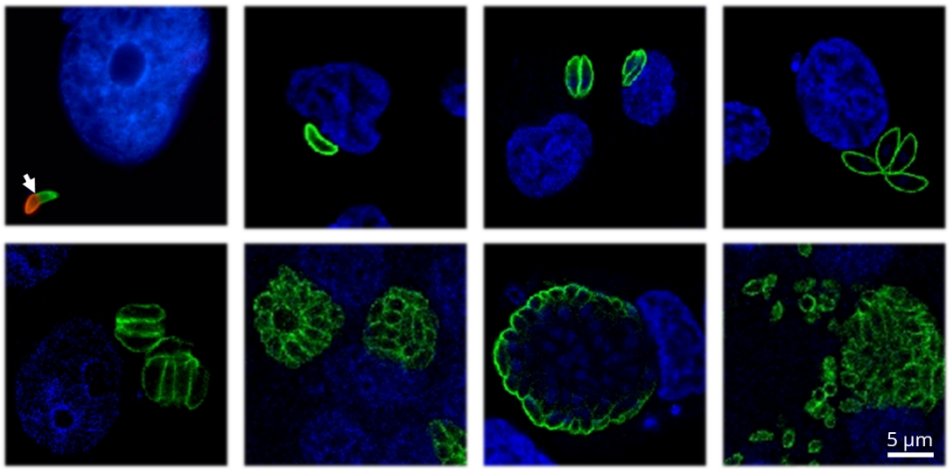


Figura 4. Representación gráfica del ciclo lítico de *N. caninum* y seguimiento del mismo en cultivo celular mediante inmunofluorescencia directa. La flecha blanca señala a un taquizoíto invadiendo una célula hospedadora.

2.2.1. Aproximación mediante deslizamiento y adhesión a la célula hospedadora

La adhesión de los taquizoítos a la célula hospedadora depende de la capacidad de los mismos para acercarse a sus dianas mediante el proceso de deslizamiento o *gliding*. En *T. gondii* el deslizamiento se compone una serie movimientos de flexión, extensión y torsión mediados por el glideosoma. En el transcurso de este proceso de locomoción, los taquizoítos desprenden rastros de la proteína de superficie

SAG1, que los hace fácilmente identificables mediante técnicas de inmunofluorescencia (Jacot *et al.*, 2014; Collantes-Fernández *et al.*, 2012; Opitz & Soldati, 2002). El deslizamiento es un fenómeno esencial para que se produzca la invasión y la egresión de los taquizoítos y que, además, parece determinar la capacidad de éstos para atravesar barreras biológicas de manera aislado-dependiente tanto en *T. gondii* (Barragan & Sibley, 2002; Dobrowolski & Sibley, 1996) como en *N. caninum* (Collantes-Fernández *et al.*, 2012).

Tras el deslizamiento el taquizoíto alcanza a la célula y establece con ella un contacto de baja afinidad a través de las proteínas de superficie SRS. En concreto, las proteínas NcSAG1 y NcSRS2 se han asociado a este fenómeno en *N. caninum* (Howe *et al.*, 1998; Sonda *et al.*, 1998; Hemphill & Gottstein, 1996). El contacto entre parásito y célula hospedadora es reversible y permite al primero asegurar la infección en células completamente receptivas, al menos en *T. gondii* (Carruthers & Boothroyd, 2007). En este sentido, un estudio reciente ha atribuido que las diferencias en el espectro de hospedadores existentes entre *T. gondii* y *N. caninum* podrían estar relacionadas con el hecho de que su perfil de expresión de proteínas SRS es diferente (Reid *et al.*, 2012).

2.2.2. Reorientación del taquizoíto y fijación apical

Una vez adheridos a la célula hospedadora, los taquizoítos se reorientan de forma perpendicular a ésta y establecen un anclaje más fuerte mediante la secreción de proteínas MIC. Este fenómeno asegura la invasión mediante la instauración de numerosas uniones entre el parásito y la célula (Carruthers & Tomley, 2008). En *N. caninum* se produce la secreción de diversas proteínas MIC a 37 ºC (Keller *et al.*, 2002; Naguleswaran *et al.*, 2001) (Tabla 1), lo que explica la pérdida de capacidad de invasión que sufren los taquizoítos tras mantenerlos extracelularmente en ausencia de células hospedadoras (Hemphill & Gottstein, 2006; Naguleswaran *et al.*, 2003). Dicha secreción podría estar asociada a un incremento de los niveles de calcio intracelular, tal y como se ha descrito para las proteínas TgMIC (Blader & Saeij, 2009; Carruthers & Sibley, 1999).

Hasta el momento se han descrito hasta seis dominios adhesivos presentes en las proteínas TgMIC (*thrombospondin-1 type 1*, *Von Willebrand A/integrin inserted (I)*, *EGF-like*, *lectin* y *microneme adhesive repeat*) que establecen interacciones proteína-proteína o proteína-carbohidrato entre el parásito y la célula hospedadora (Friedrich *et al.*, 2010; Carruthers & Tomley, 2008). Algunos de estos dominios han sido también descritos en las proteínas NcMIC, por lo que cabría esperar que ambas se unieran a ligandos similares (Keller *et al.*, 2004; Keller *et al.*, 2002; Lovett *et al.*, 2000; Sonda *et al.*, 2000). No obstante, existen evidencias experimentales que demuestran lo contrario. Por un lado, se ha observado que las proteínas TgMIC y NcMIC se unen a diferentes tipos de glucosaminoglicanos de la superficie de la célula hospedadora; mientras que las proteínas TgMIC se unen preferencialmente a residuos de heparán-sulfato y ácido siálico (Friedrich *et al.*, 2010), las NcMIC optan por residuos de condroitín-sulfato (Naguleswaran *et al.*, 2002). Por otro lado, los dominios adhesivos *lectin-like* sólo han sido descritos en las proteínas TgMIC

(Friedrich *et al.*, 2010). Además, las proteínas NcMIC19 y NcMIC26 sólo se han descrito en *N. caninum*, sin que exista homología con ninguna otra proteína de *T. gondii* (Reid *et al.*, 2012). Estas diferencias, sumadas a las descritas para otras proteínas que participan en el ciclo lítico, podrían justificar el diferente espectro de hospedadores de *N. caninum* y *T. gondii*.

La importancia de la interacción de las proteínas de los micronemas con la superficie de la célula hospedadora se ha demostrado *in vitro* mediante ensayos de inhibición de la invasión empleando anticuerpos específicos frente a diversas proteínas NcMIC (Naguleswaran *et al.*, 2001; Nishikawa *et al.*, 2000c; Hemphill *et al.*, 1996). Además, su utilidad vacunal también se ha demostrado en el modelo murino (Monney *et al.*, 2012; Monney *et al.*, 2011; Debache *et al.*, 2010; Debache *et al.*, 2009; Cannas *et al.*, 2003b; Nishikawa *et al.*, 2002; Nishikawa *et al.*, 2001a). Estas proteínas son ricas en residuos de cisteína (Keller *et al.*, 2004; Keller *et al.*, 2002; Sonda *et al.*, 2000), por lo que su conformación espacial depende del establecimiento de puentes disulfuro por parte de la proteína NcPDI (*Protein disulfide isomerase*), presente también en los micronemas y en la superficie del parásito (Naguleswaran *et al.*, 2005), y que también ha sido evaluada como vacuna en el modelo murino (Debache & Hemphill, 2013).

Proteína	Localización	Función	Referencia
NcMIC1	Micronemas	Adhesión	(Keller *et al.*, 2002)
NcMIC2	Micronemas	Adhesión	(Lovett *et al.*, 2000)
NcMIC3	Micronemas, superficie	Adhesión	(Naguleswaran *et al.*, 2001; Sonda *et al.*, 2000)
NcMIC4	Micronemas	Adhesión	(Keller *et al.*, 2004)
NcMIC6	Micronemas	-	(Li *et al.*, 2015)
NcMIC10	-	-	(Atkinson *et al.*, 2001)
NcMIC11	-	-	(Harper *et al.*, 2004)
NcMIC17	Micronemas	Adhesión	(Sohn *et al.*, 2011)
NcAMA1	Apical	Invasión	(Zhang *et al.*, 2007)
NcRON2	-	-	(Marugán-Hernández *et al.*, 2011a)
NcRON3	-	-	(Marugán-Hernández *et al.*, 2011a)
NcRON4	Cuello de roptrias	-	(Marugán-Hernández *et al.*, 2011a; Sohn *et al.*, 2011)
NcRON8	Cuello de roptrias	-	(Marugán-Hernández *et al.*, 2011a; Sohn *et al.*, 2011)
NcROP1	VP	-	(Marugán-Hernández *et al.*, 2011a)
NcROP2Fam-1	Roptrias y evacuolas	Invasión	(Alaeddine *et al.*, 2013; Debache *et al.*, 2009; Debache *et al.*, 2008)
NcROP4	Roptrias	-	(Sohn *et al.*, 2011)
NcROP5	-	-	(Marugán-Hernández *et al.*, 2011a)
NcROP8	-	-	(Marugán-Hernández *et al.*, 2011a)
NcROP9	Roptrias	-	(Marugán-Hernández *et al.*, 2011a; Sohn *et al.*, 2011)
NcROP30	Roptrias	-	(Marugán-Hernández *et al.*, 2011a)
NcGRA1	GD, quiste	-	(Vonlaufen *et al.*, 2004; Atkinson *et al.*, 2001)
NcGRA2	GD, quiste	-	(Vonlaufen *et al.*, 2004; Ellis *et al.*, 2000)
NcGRA6	GD, superficie	Invasión	(Cho *et al.*, 2005; Liddell *et al.*, 1998)
NcGRA7	GD, VP, quiste	Invasión	(Aguado-Martínez *et al.*, 2010; Lally *et al.*, 1997)
NcGRA9	GD, bradizoíto	-	(Marugán-Hernández *et al.*, 2010)
NcGRA14	GD	-	(Liu *et al.*, 2013)
NcMAG1	GD, VP, quiste	-	(Guionaud *et al.*, 2010)
NcNTPasa I	GD	Actividad enzimática	(Asai *et al.*, 1998)

Tabla 1. Proteínas de micronemas, roptrias y gránulos densos identificadas en los taquizoítos de *N. caninum*. VP: vacuola parasitófora. GD: gránulos densos.

2.2.3. Invasión de la célula hospedadora

La invasión de la célula hospedadora es un proceso muy conservado entre los parásitos apicomplejos (Straub *et al.*, 2009) y ha sido ampliamente estudiada en *T. gondii* (Mital *et al.*, 2005). Durante el proceso de invasión, los taquizoítos forman una estructura anular, la unión móvil, que sirve de puerta de entrada hacia la célula hospedadora. La unión móvil está formada por la proteína de micronemas TgAMA1 y por las proteínas del cuello de las roptrias TgRON2, TgRON4, TgRON5 y TgRON8. Todas ellas son secretadas durante la invasión y forman complejos entre sí que interaccionan estrechamente con la membrana de la célula hospedadora y con ciertas proteínas del glideosoma del parásito (Beck *et al.*, 2014). En *N. caninum* se ha demostrado la existencia de las proteínas NcAMA1, NcRON2, NcRON4, NcRON5 y NcRON8, por lo que el proceso de invasión podría ser similar al descrito para *T. gondii* (Marugán-Hernández *et al.*, 2011a; Straub *et al.*, 2009; Zhang *et al.*, 2007) (Tabla 1).

Una vez establecida la unión móvil, el taquizoíto se internaliza progresivamente y de forma activa hacia el citoplasma de la célula hospedadora por medio de un estrangulamiento de la membrana de la misma promovido por el glideosoma del parásito. Al mismo tiempo, la unión móvil se desliza desde el ápice del parásito hasta su polo más caudal, descartando de forma selectiva aquellas proteínas del hospedador presentes en la membrana plasmática que el parásito arrastra durante la invasión. De este modo, se impide que la futura vacuola parasitófora se funsione con los lisosomas del hospedador y sea destruida (Alexander *et al.*, 2006; Alexander *et al.*, 2005; Beyer *et al.*, 2002; Mordue *et al.*, 1999a; Mordue *et al.*, 1999b).

2.2.4. Formación y maduración de la vacuola parasitófora

En *T. gondii*, la formación de la vacuola parasitófora depende de la actividad de diversas proteasas del parásito que actúan desde el establecimiento de la unión móvil. La acción de dichas proteasas permite que el taquizoíto vaya siendo englobado de forma progresiva en una vacuola formada a partir de la membrana del hospedador (Carruthers & Boothroyd, 2007; Mordue & Sibley, 1997). Curiosamente, el proceso de invasión, pero no la adhesión, puede ser bloqueado con citocalasina D tanto en *T. gondii* (Carruthers & Boothroyd, 2007; Keeley & Soldati, 2004; Soldati & Meissner, 2004), como en *N. caninum* (Hemphill *et al.*, 1996). Este compuesto bloquea la polimerización de los filamentos de actina y evita su elongación, lo que enlazaría el proceso de invasión con cambios en la conformación del citoesqueleto del parásito. Análogamente, *T. gondii* precisa de una remodelación del citoesqueleto de la célula hospedadora en el transcurso de la invasión para facilitar el desarrollo de la vacuola parasitófora (Delorme-Walker *et al.*, 2012; Gonzalez *et al.*, 2009) . En conjunto, estos hechos subrayan la importancia del glideosoma en el proceso de invasión.

Las proteínas de las roptrias y de los gránulos densos se consideran importantes factores de virulencia de *T. gondii*, puesto que son inyectadas al interior de la célula hospedadora durante la invasión y formación de la vacuola parasitófora (Qiu *et al.*, 2009; Boothroyd & Dubremetz, 2008; Bradley & Sibley, 2007). Muchas de estas proteínas son liberadas en forma de vacuolas no membranosas (evacuolas) que se distribuyen a por el citoesqueleto de la célula hospedadora. Entre ellas destacan las proteínas TgROP2, TgROP4, TgROP7 y TgGRA7 (Dunn *et al.*, 2008; El Hajj *et al.*, 2007b; Carey *et al.*, 2004). Las proteínas ROP pertenecientes a la familia ROP2 han sido las mejor estudiadas en *T. gondii*. Todas ellas comparten tres características comunes: se secretan durante la invasión de la célula hospedadora, se asocian a la membrana de la vacuola parasitófora o migran al núcleo y presentan un dominio quinasa C-terminal (El Hajj *et al.*, 2006). Concretamente, las proteínas TgROP16, TgROP18 y TgROP38 son capaces de remodelar la transcripción de ciertos genes de la célula hospedadora a través de su dominio quinasa. TgROP18 participa en la inactivación de IRGs (*Immunity-Related GTPases*) en el ratón, que son responsables de la destrucción de la vacuola parasitófora en respuesta al IFN-γ (Fleckenstein *et al.*, 2012; Fentress & Sibley, 2011), TgROP16 modula la respuesta inmunitaria innata modificando a nivel nuclear los factores de transcripción STAT3 y STAT6 (Schneider *et al.*, 2013; Hunter & Sibley, 2012) y TgROP38 altera la ruta de señalización MAPK que controla la apoptosis y la proliferación celular (Peixoto *et al.*, 2010). No obstante, muchos otros miembros de la familia ROP2 presentan dominios catalíticamente inactivos (Talevich & Kannan, 2013). Pese a ello, la mayoría son secretadas en la célula hospedadora, lo que sugiere que están involucradas en algún mecanismo de patogenicidad del parásito (Reese & Boyle, 2012). Este es el caso de TgROP5, una pseudoquinasa implicada en la activación de TgROP18 (Behnke *et al.*, 2015; Behnke *et al.*, 2012; Fleckenstein *et al.*, 2012; Behnke *et al.*, 2011).

En contraste, la información disponible sobre las proteínas ROP de *N. caninum* es muy limitada (Tabla 1). NcROP2Fam-1 es la única proteína caracterizada hasta la fecha, habiéndose demostrado su participación en la invasión y su secreción en forma de evacuolas (Alaeddine *et al.*, 2013). No obstante, ya sea mediante estudios proteómicos o mediante inmunodetección por anticuerpos monoclonales dirigidos frente proteínas de roptrias de *T. gondii*, se han descrito numerosas proteínas NcROP que comparten un elevado grado de homología con sus respectivas proteínas ortólogas en *T. gondii* (Marugán-Hernández *et al.*, 2011a; Sohn *et al.*, 2011). Por otro lado, y mediante un abordaje *in silico*, se ha establecido una clasificación de las proteínas de la familia ROP2 en base a las características de sus dominios quinasa, que en muchos casos son compartidas entre proteínas ortólogas en *T. gondii* (Talevich & Kannan, 2013). Además, y a pesar de estas similitudes, se ha demostrado la existencia de cierta divergencia entre los genes que codifican las proteínas ROP de *T. gondii* y *N. caninum* mediante estudios transcriptómicos. En concreto, *ROP18* es un pseudogen, y el número de copias de *ROP5* es menor en *N. caninum* (Reid *et al.*, 2012). Curiosamente, la incorporación del gen Tg*ROP18* a una cepa transgénica de *N. caninum* se ha asociado recientemente a un incremento de la proliferación intracelular del parásito y a una mayor virulencia *in vivo* en ratones (Lei *et al.*, 2014).

Al igual que con las proteínas ROP, el número de trabajos disponibles dedicados a las proteínas de gránulos densos de *T. gondii* es mucho más numeroso que en *N. caninum*. Éstas pueden secretarse al espacio intravacuolar (Cy-18, TgPI1, TgPI2, TgPL1, TgGRA1 y TgGRA2), incluirse en la membrana de la vacuola parasitófora (TgGRA3, TgGRA5, TgGRA7, TgGRA8, TgGRA10 y TgGRA14) o localizarse en la red túbulovesicular (TgGRA2, TgGRA3, TgGRA4, TgGRA6, TgGRA7, TgGRA9, TgGRA12, TgGRA14 y TgNTPasa) (Okada *et al.*, 2013). En conjunto, la modificación de la membrana de la vacuola y el establecimiento de una red túbulovesicular aseguran la supervivencia y nutrición de los parásitos y aportan un ambiente favorable para su replicación (Nam, 2009; Mercier *et al.*, 2005; Dubremetz *et al.*, 1993). Además, las proteínas de gránulos densos parecen estar involucradas en procesos de inmunomodulación (TgGRA15, TgGRA24, TgGRA25) (Shastri *et al.*, 2014; Rosowski *et al.*, 2011), de alteración de la expresión génica (TgGRA16) (Braun *et al.*, 2013), de asociación con la mitocondria del hospedador (TgMAF1) (Pernas *et al.*, 2014) y en la propia egresión del parásito (TgGRA22) (Okada *et al.*, 2013).

El número de proteínas de gránulos densos que han sido descritas hasta el momento en *N. caninum* es mucho menor (Tabla 1). NcGRA6 y NcGRA7 han sido relacionadas con los procesos de invasión del taquizoíto (Aguado-Martínez *et al.*, 2010; Cho *et al.*, 2005), aunque se desconoce la función del resto de las proteínas descritas. La mayoría son secretadas a la vacuola parasitófora, llegando a formar, en algunos casos, parte de los quistes tisulares típicos de la fase crónica de la infección (Guionaud *et al.*, 2010; Cho *et al.*, 2005; Vonlaufen *et al.*, 2004). No obstante, el factor de virulencia Tg*GRA15* es un pseudogen en *N. caninum*, del mismo modo que no se ha identificado el ortólogo del gen Tg*GRA24* (English *et al.*, 2015; Reid *et al.*, 2012). Por otro lado, se ha determinado la existencia de la proteína NcNTPasa-I. De ella sólo se sabe que posee actividad hidrolasa y que podría presentar diversas isoformas, aunque su función concreta no ha sido completamente descifrada (Asai *et al.*, 1998). Curiosamente, se ha descrito una asociación entre las isoformas de la NTPasa que expresa *T. gondii* y la virulencia de la cepa (Johnson *et al.*, 2003).

Cabe destacar que la mayoría de los experimentos de caracterización de las proteínas involucradas en el ciclo lítico de *T. gondii* se han fundamentado en experimentos de interrupción de su expresión mediante la creación de parásitos *knock out* o a través de técnicas de ARN de interferencia. Por el contrario, estos métodos no se han llevado a cabo hasta la fecha en *N. caninum*.

2.2.5. Egresión

La egresión es la fase final del ciclo lítico y conlleva la liberación de los parásitos de un compartimento intracelular en el que los recursos necesarios para su supervivencia son escasos. Este fenómeno es el responsable directo de las lesiones presentes en el hospedador, que son consecuencia de la lisis celular y de la reacción inmunitaria que se desencadena localmente. El proceso de egresión parece

estar estrechamente regulado y coordinado con el proceso de invasión de nuevas células. De este modo, se asegura que el parásito pase el menor tiempo posible en el inhóspito ambiente extracelular.

El incremento de los niveles intracelulares de calcio parece ser esencial para inducir la egresión en *T. gondii* (Millholland *et al.*, 2013), puesto que determina la activación de quinasas (TgCDPK1 y 3, TgPKG) y metil-transferasas (TgAKMT) imprescindibles para la consecución del proceso (Heaslip et al., 2010; (Garrison *et al.*, 2012; Lourido *et al.*, 2012; McCoy *et al.*, 2012; Heaslip *et al.*, 2011). La egresión también depende de la activación del deslizamiento de los taquizoítos y de la secreción de la proteína TgPL1 (*Perforin-like*) de micronemas, que desorganiza la membrana y facilita la salida de los parásitos (Roiko & Carruthers, 2013; Kafsack *et al.*, 2009; Lavine & Arrizabalaga, 2008). De hecho, el glideosoma y los micronemas se encuentran íntimamente relacionados en el taquizoíto (Blackman & Carruthers, 2013). Además, el proceso de egresión parece depender de una perturbación en el citoesqueleto de la célula hospedadora (Chandramohanadas *et al.*, 2009). Por otro lado, las proteínas de la familia CDPK (*Calcium dependent protein-kinases*) se encargan de regular mediante fosforilación dependiente de las concentraciones de calcio intracelular los procesos de invasión, deslizamiento y secreción de proteínas de micronemas, constituyéndose como importantes dianas terapéuticas (Keyloun *et al.*, 2014). En cuanto a las roptrias, éstas no parecen ser esenciales para la egresión del parásito (Beck *et al.*, 2013; Mueller *et al.*, 2013).

2.3. Persistencia y reactivación de la infección

Tras la sucesión de varios ciclos líticos, el hospedador infectado desarrolla una respuesta inmunitaria específica frente al parásito que es capaz de eliminar a la mayoría de los taquizoítos (Weiss *et al.*, 1999). Los procesos inmunológicos que parecen liderar el control de la infección son la actividad de las células T CD4+ y CD8+, la inhibición de la multiplicación del parásito mediada por la secreción de IFN-γ y TNF-α, y el bloqueo de la invasión a través de anticuerpos (Innes, 2007). Análogamente, la secreción de IFN-γ, TNF-α e IL-6 se ha relacionado con la inducción de la conversión a bradizoíto en *T. gondii*. Concretamente, la presencia de IFN-γ parece mediar la liberación de metabolitos reactivos como el NO (Bohne et al., 1994) y privar a los taquizoítos de aminoácidos esenciales como la arginina y el triptófano (Fox *et al.*, 2004; Bohne *et al.*, 1994; Pfefferkorn *et al.*, 1986). Igualmente, el IFN-γ activa IRGs, que participan en la destrucción de la vacuola parasitófora en macrófagos (Butcher *et al.*, 2005).

La conversión a bradizoíto permite evadir la respuesta inmunitaria y mantener al hospedador crónicamente infectado. En *N. caninum* este proceso es rápido y asincrónico (Risco-Castillo *et al.*, 2004) y se acompaña de importantes cambios en el perfil de expresión proteico. Entre otros, destacan la expresión proteínas específicas de estadio (NcSRS9, NcSAG4 y NcBSR4) (Risco-Castillo *et al.*, 2011; Aguado-Martínez *et al.*, 2009b; Risco-Castillo *et al.*, 2007), así como de otras proteínas que intervienen en el metabolismo anaeróbico, en las respuestas al estrés y en la reparación del ADN (Marugán-Hernández *et*

al., 2010). En general, la conversión a bradizoíto parece ser un proceso muy conservado, puesto que *T. gondii* se comporta de forma similar. El desarrollo de esta forma de latencia se ha asociado a diversas respuestas ante el estrés, que determinan la fosforilación del factor de transcripción TgIF2α (Sullivan & Jeffers, 2012; Narasimhan *et al.*, 2008; Sullivan *et al.*, 2004). Este factor parece controlar muchos de los cambios transcripcionales asociados al proceso de conversión, como son el aumento de expresión de ciertas proteínas relacionadas con las respuestas al estrés, el metabolismo anaerobio o la depuración de metabolitos reactivos (Sullivan & Jeffers, 2012).

Los bradizoítos se acantonan dentro de la vacuola parasitófora, que acaba por convertirse en un quiste con una pared bien instaurada (Beyer *et al.*, 2002). Tanto en *N. caninum* como en *T. gondii* los quistes se localizan preferentemente en órganos inmunoprivilegiados como el sistema nervioso central, aunque también pueden detectarse en musculatura esquelética (Dubey *et al.*, 2006; Peters *et al.*, 2001; Luder *et al.*, 1999; Dubey *et al.*, 1998b). Allí evitan la exposición del parásito a la respuesta inmunitaria y favorecen la cronificación de la infección (Innes *et al.*, 2002). Al contrario de lo que se piensa, los bradizoítos de *T. gondii* presentan una elevada movilidad dentro del quiste, e incluso han demostrado ser capaces de abandonarlo sin producir su rotura, lo que determina un mecanismo de transmisión adicional durante la fase crónica de la enfermedad (Dzierszinski *et al.*, 2004). Durante esta fase el hospedador infectado no muestra signos clínicos y puede llegar a mantenerse infectado de por vida (Dubey & Lindsay, 1996). Sin embargo, la persistencia de quistes de *T. gondii* en ratas y ratones inmunocompetentes se ha asociado a ciertos cambios comportamentales que favorecerían la transmisión del parásito al hospedador definitivo (Vyas *et al.*, 2007a; Vyas *et al.*, 2007b; Webster, 2007). El proceso de conversión a bradizoíto es reversible, pudiéndose reactivar una infección latente e iniciarse una nueva oleada de ciclos de proliferación del taquizoíto en el organismo. Los mecanismos que modulan esta reversión no han sido determinados, aunque parece que la respuesta inmunitaria juega un papel muy importante. La reactivación de la infección se ha asociado a estados de inmunosupresión, ya sea secundarios o inducidos mediante corticoesteroides (Pereira-Chioccola *et al.*, 2009; Nicoll *et al.*, 1997). Esto sugiere que el parásito requiere de cierta presión del sistema inmune para mantener su latencia, y que ésta desaparece en el momento que la respuesta inmunitaria falla. De acuerdo a esta hipótesis, la secreción de IFN-γ parece clave para mantener la infección latente (Dunay *et al.*, 2009). Sin embargo, la reversión del estadio de bradizoíto al de taquizoíto parece ocurrir periódicamente en el hospedador infectado (Eaton *et al.*, 2006). La reactivación de la infección por *N. caninum* parece seguir los mismos preceptos que en *T. gondii*. Los estados de inmunosupresión se han asociado a la recrudescencia de la enfermedad (Rettigner *et al.*, 2004a; Quinn *et al.*, 2002a), especialmente durante la gestación. Dado que este periodo va acompañado de una importante modulación inmunitaria para prevenir el rechazo del feto por parte de la madre, el parásito podría encontrar un ambiente idóneo transformarse de nuevo al estadio de taquizoíto, proliferar y alcanzar el feto verticalmente a través de la placenta (Quinn *et al.*, 2004).

3. Desarrollo de vacunas frente a la neosporosis bovina

Neospora caninum presenta una íntima adaptación a sus hospedadores que obstaculiza el desarrollo de vacunas frente a la enfermedad que produce. Por un lado, el taquizoíto cuenta con un ciclo de vida estrictamente intracelular que dificulta su eliminación por parte del sistema inmunitario. Por otro lado, el parásito es capaz de sufrir una conversión al estadio de bradizoito y acantonarse en el interior de quistes tisulares, lo que le permite evadir la respuesta inmunitaria. Además, cabe destacar que los hospedadores infectados por *N. caninum* desarrollan un cierto grado de inmunidad concomitante. Dicha inmunidad les protege frente a reinfecciones, aunque éstos resultan incapaces de erradicar la infección de origen. Pese a todo lo citado, la posibilidad de desarrollar vacunas que prevengan la transmisión vertical del parásito parece factible por dos causas: las hembras gestantes e infectadas de modo exógeno presentan tasas de transmisión vertical más bajas y las hembras crónicamente infectadas ven reducida dicha tasa con la edad (Dijkstra *et al.*, 2008; Dijkstra *et al.*, 2003; Romero *et al.*, 2002). Todo ello indica que el sistema inmunitario sería capaz de controlar la diseminación del parásito desde la madre al feto bajo determinadas circunstancias y mediante mecanismos pendientes de dilucidar.

Idealmente, una vacuna frente a *N. caninum* en el ganado bovino debería prevenir la transmisión vertical y evitar la cronificación de la enfermedad. Además, en el caso del perro, una vacuna ideal debería reducir la liberación de ooquistes en las heces. El desarrollo de vacunas requiere de un extenso conocimiento de la respuesta inmunitaria que desencadena el parásito en el transcurso de la infección y de los adyuvantes disponibles para su formulación. De tal modo, la combinación de un adyuvante adecuado con uno o varios antígenos vacunales específicos podría desencadenar respuestas inmunitarias de base celular y humoral, tanto a nivel sistémico como a nivel de las mucosas, que son esenciales para el control la enfermedad (Monney & Hemphill, 2014). Igualmente, el desarrollo de nuevas vacunas depende del empleo de modelos animales armonizados que permitian obtener datos certeros tanto de la eficacia como de la seguridad de una vacuna en desarrollo.

3.1. Respuesta inmunitaria frente a *N. caninum*

3.1.1. Respuesta inmunitaria en el ganado bovino

La infección por *N. caninum* en el ganado bovino desencadena una combinación de mecanismos de inmunidad innata y adquirida que, en conjunto, controlan la diseminación del parásito por el organismo, al menos experimentalmente (Regidor-Cerrillo *et al.*, 2014; Bartley *et al.*, 2013; Rosbottom *et al.*, 2008; Andrianarivo *et al.*, 2005; Almería *et al.*, 2003; Staska *et al.*, 2003; Innes *et al.*, 2001b). Las células *natural killer* (NK) actúan como primera línea de defensa, ya que destruyen las células infectadas por *N. caninum* y liberan IFN-γ (Klevar *et al.*, 2007; Boysen *et al.*, 2006). Por otro lado, el papel de los TLR (*Toll-like*

receptors) podría ser clave durante las fases iniciales de la infección, ya que se encuentran presentes en las células presentadoras de antígeno (TLR2), así como en los linfocitos B (TLR9) (Werling *et al.*, 2006). No obstante, en las etapas tempranas de la infección no existe una tendencia clara hacia respuestas inmunitarias de tipo celular (Th1) o humoral (Th2), puesto que se secretan cantidades significativas de IFN-γ e IL-4 (Regidor-Cerrillo *et al.*, 2014; Rosbottom *et al.*, 2007).

A medida que avanza la infección se produce una polarización de la respuesta inmunitaria hacia una respuesta de tipo Th1 en la que predomina la secreción de IFN-γ sobre la de IL-4, así como los isotipos IgG2 sobre los IgG1 (Regidor-Cerrillo *et al.*, 2014; Caspe *et al.*, 2012; Rojo-Montejo *et al.*, 2009a; Gibney *et al.*, 2008; Andrianarivo *et al.*, 2001; Williams *et al.*, 2000). El IFN-γ activa a las células T CD4+, que a su vez estimula a las células T CD8+, también capaces de secretar IFN-γ (Rosbottom *et al.*, 2007; Maley *et al.*, 2006). En conjunto, estos componentes inmunitarios destruyen a la mayoría de las células infectadas por el parásito, inhiben su proliferación por medio de la secreción de IFN-γ y TNF-α y bloquean la invasión de nuevas células a través de los anticuerpos generados frente a los taquizoítos (Innes, 2007). En este proceso, la secreción de IL-17 por parte de las células T CD4+ previamente activadas por macrófagos también parece jugar un papel clave en el control de la infección (Flynn & Marshall, 2011). La respuesta inmunitaria descrita es capaz de reducir drásticamente el número de taquizoítos diseminados por el organismo, quedando únicamente los bradizoítos, que se localizan en tejidos inmunoprivilegiados.

El papel de la respuesta inmunitaria humoral frente a *N. caninum* es todavía confuso. Los niveles de anticuerpos frente a los taquizoítos sufren fluctuaciones a lo largo de la gestación (Nogareda *et al.*, 2007; Andrianarivo *et al.*, 2005; Guy *et al.*, 2001; Quintanilla-Gonzalo *et al.*, 1999; Stenlund *et al.*, 1999), lo que puede tener relación con la propia actividad del parásito (Innes, 2007; Weston *et al.*, 2005). Por otro lado, se ha descrito un cierto desarrollo de inmunidad frente a la infección tras gestaciones sucesivas en hembras crónicamente infectadas, lo cual se refleja en el desarrollo de su respuesta humoral (Benavides *et al.*, 2012; Nogareda *et al.*, 2007; Sager *et al.*, 2001). Sin embargo, el incremento del título de anticuerpos específicos ha sido asociado a un mayor riesgo de aborto (Brickell *et al.*, 2010; Kashiwazaki *et al.*, 2004; Pereira-Bueno *et al.*, 2003). No obstante, la protección frente al aborto parece ir asociada a unos niveles elevados de IgG2, aunque exclusivamente en ganado de carne, donde se produce una elevada producción de IFN-γ (Santolaria *et al.*, 2011; Almería *et al.*, 2009).

A pesar de ser efectiva, la respuesta inmunitaria puede resultar perjudicial para la placenta y el feto cuando se produce en una hembra gestante (Almería *et al.*, 2010; Rosbottom *et al.*, 2008; Maley *et al.*, 2006; Innes *et al.*, 2005; Quinn *et al.*, 2002b). De hecho, durante la gestación se produce una modulación fisiológica materna hacia respuestas del tipo Th2 con las que se disminuye el riesgo de rechazo del feto (Entrican, 2002). Por el contrario, el control de la neosporosis parece depender principalmente de respuestas del tipo Th1. No obstante, aunque la producción periférica de IFN-γ se ha asociado a la protección frente al aborto (Almería *et al.*, 2012; Bartley PM *et al.*, 2012; Almería *et al.*, 2009; Lopez-Gatius

et al., 2007), ciertos estudios señalan lo contrario (Regidor-Cerrillo *et al.*, 2014). Esto indica que el IFN-γ tendría una función protectora hasta un cierto nivel y que una vez superado iría en detrimento de la viabilidad fetal (Innes, 2007; Innes *et al.*, 2005; Entrican, 2002). Por otro lado, la secreción de IL-10, IL-4 y TGF-β se ha asociado a un menor daño de la placenta y del feto por contrarrestar el efecto del IFN-γ, pero también a una mayor replicación del parásito y a un incremento de la tasa de transmisión transplacentaria (Innes, 2007; Entrican, 2002; Innes *et al.*, 2000). Por lo tanto, las consecuencias de la infección por *N. caninum* dependen de un correcto balance Th1/Th2 que sea lo suficientemente eficaz para bloquear al parásito, pero sin producir daño fetal.

El estado de desarrollo de la respuesta inmunitaria del feto en el momento de la infección es otro factor clave que determina las consecuencias de la misma. En este sentido, las infecciones durante el primer tercio de gestación se asocian a lesiones más graves, mientras que durante el segundo y tercer tercio de gestación las lesiones que se observan son leves (Gibney *et al.*, 2008; Collantes-Fernández *et al.*, 2006c; Macaldowie *et al.*, 2004). Estas diferencias se deben al desarrollo de la respuesta inmunitaria del propio feto, que es detectable desde las 12-15 semanas de gestación, y que sería capaz de combatir la infección por sí misma cuando los taquizoítos alcanzaran los tejidos fetales (Regidor-Cerrillo *et al.*, 2014; Bartley PM *et al.*, 2012; Innes *et al.*, 2005).

3.1.2. Respuesta inmunitaria en el ratón

El conocimiento de la respuesta inmunitaria en el ratón tras la infección por *N. caninum* es muy amplio debido a su empleo como modelo animal. Al igual que en el ganado bovino, el control de la infección depende de una combinación de las respuestas inmunitarias humoral y celular (Hemphill *et al.*, 2006). *N. caninum* es capaz de infectar macrófagos y células dendríticas, donde altera la expresión de marcadores de superficie (Dion *et al.*, 2011). Estas células secretan IL-12, TNF-α e IL-10 en las fases iniciales de la infección, dando lugar a respuestas inmunitarias mixtas de tipo celular y humoral (Feng *et al.*, 2010; Strohbusch *et al.*, 2009). No obstante, la secreción de IL-12 activa a las células T CD4+, que acaban desviando la respuesta hacia una de tipo celular, con secreción de IFN-γ y activación de linfocitos T CD8+ que limitan la diseminación del parásito (Spencer *et al.*, 2005; Ritter *et al.*, 2002; Nishikawa *et al.*, 2001b; Khan *et al.*, 1997). Sin embargo, esta polarización de la respuesta no parece presentarse cuando las células dendríticas son estimuladas con lisados de parásito, en cuyo caso se produce una importante secreción de IL-4 (Feng *et al.*, 2010). Las células NK y NKT (*Natural Killer T-cells*) son otros importantes componentes de la respuesta inmunitaria innata frente a *N. caninum* en el ratón, ya que también participan en la activación de los linfocitos T CD4+ (Nishikawa *et al.*, 2010).

Los receptores que parecen mediar estas respuestas innatas son los TLR2 y 3. Por un lado, los ratones TLR2(-/-) son extremadamente susceptibles a la infección experimental por el parásito, mostrando una baja proliferación de los linfocitos T CD4+ y CD8+ y una baja secreción de IFN-γ (Mineo *et al.*, 2010). Por otro lado, el receptor TLR3 también parece estar implicado en la respuesta innata frente a *N. caninum*. Este receptor se ha asociado tradicionalmente a la respuesta inespecífica frente a virus ARN y, recientemente, ha demostrado estar implicado en la producción de interferón de clase I (α/β) por parte de las células presentadoras de antígeno ante una infección por *N. caninum*. No obstante, el IFN-γ parece ser el único capaz de controlar la multiplicación de los taquizoítos (Beiting *et al.*, 2014). En este sentido, cabe destacar que el número de TLR expresados por el ratón (TLR1-10 y 11-13) es mayor que el que expresa el ganado bovino (TLR1-10) (Novak, 2014). De hecho, se han descrito diferencias en la respuesta de un mismo receptor estimulado por un mismo ligando en diferentes especies animales (Werling *et al.*, 2009). Por otro lado, las IRGs sólo parecen estar presentes en el ratón. Estas enzimas son las responsables de la destrucción de la vacuola parasitófora en *T. gondii* tras la activación de los receptores TLR11 y 12 (Gazzinelli *et al.*, 2014). En conjunto, estos hallazgos demuestran las claras diferencias existentes entre las respuestas innatas desencadenadas por cada una de las especies animales ante un mismo agente.

Por otro lado, la capacidad de respuesta frente al parásito difiere en función de la estirpe de ratón. En general, las estirpes no consanguíneas son resistentes a la infección por *N. caninum*, mientras que las estirpes consanguíneas son mucho más susceptibles (Miller *et al.*, 2005; Collantes-Fernández *et al.*, 2004; Quinn *et al.*, 2004; Rettigner *et al.*, 2004b; Quinn *et al.*, 2002b). De hecho, se ha demostrado que las estirpes consanguíneas presentan una tendencia hacia respuestas humorales Th2, donde predomina la secreción de IL-4 sobre la de IFN-γ, y la producción de IgG1 sobre la de IgG2a (Miller *et al.*, 2005; Long & Baszler, 2000; Long *et al.*, 1998). Además, se han descrito evidentes diferencias funcionales entre la respuesta inmunitaria desarrollada por las estirpes consanguíneas más ampliamente utilizadas como modelos animales en estudios de neosporosis (Mols-Vorstermans *et al.*, 2013). En ellas, la protección frente a *N. caninum* parece estar mediada por el IFN-γ y la IL-12 (Ritter *et al.*, 2002; Nishikawa *et al.*, 2001c; Baszler *et al.*, 1999; Khan *et al.*, 1997), aunque el TNF-α, la IL-10 y el TGF-β parecen ser clave, al menos *in vitro* (Jesus *et al.*, 2013).

Los ratones de la estirpe BALB/c han sido uno de los más utilizados como modelos murinos de neosporosis cerebral y congénita por su elevada susceptibilidad a la infección por *N. caninum*. Esta estirpe presenta una expansión de la población de linfocitos B tras la infección, con una producción predominante de inmunoglobulinas del tipo IgG2a (Teixeira *et al.*, 2005). En ella las células T CD4+ parecen ser las principales responsables de la protección frente al parásito, ya que en las fases más avanzadas de la infección son las únicas que prevalecen (Tanaka *et al.*, 2000). Al igual que ocurre durante la gestación en el ganado bovino, las ratonas gestantes expresan principalmente citoquinas que fomentan respuestas de tipo Th2 para evitar el rechazo de los fetos, tanto a nivel sistémico como localmente en la placenta (Raghupathy, 1997; Lin *et al.*, 1993; Wegmann *et al.*, 1993). Este ambiente resulta más permisivo para la

replicación activa del parásito y su transmisión vertical (Kano *et al.*, 2005; Quinn *et al.*, 2004; Long & Baszler, 2000). Sin embargo, el control de la transmisión vertical parece estar controlado por respuestas Th1 y Th2 (Haldorson *et al.*, 2005; Long & Baszler, 2000), por lo que la regulación inmunológica podría ser más compleja de lo que se conoce hasta el momento. Ante una infección por *N. caninum*, la expresión de citoquinas placentarias es variable en función del tercio de gestación en que se produzca. Durante el primer y último tercio se ha descrito un incremento de la expresión de IFN-γ, TNF-α e IL-10 (López-Pérez *et al.*, 2011), mientras que en el segundo tercio sólo se sobre-expresan el TNF-α y la IL-4 (López-Pérez *et al.*, 2010). La presencia de IFN-γ y TNF-α localmente durante el primer y último tercio de la gestación se ha asociado a una mayor mortalidad por daño placentario (López-Pérez *et al.*, 2008). Del mismo modo, el efecto antagonista de la IL-4 sobre el IFN-γ durante el segundo tercio de la gestación podría ser la causa de la elevada transmisión vertical que se ha observado durante este periodo (López-Pérez *et al.*, 2006).

3.2. Vacunas frente a la neosporosis bovina

Puesto que la vacunación se ha presentado como una medida económicamente rentable para el control de la neosporosis bovina, el número de estudios dedicados a dicho aspecto ha sufrido un importante incremento durante los últimos 16 años gracias a la implantación de diversos modelos animales y al creciente conocimiento de las interacciones entre el parásito y el hospedador (Reichel *et al.*, 2014; Reichel *et al.*, 2013). El desarrollo de una vacuna frente a la neosporosis bovina debería cumplir dos requisitos: ser capaz de prevenir la transmisión transplacentaria endógena y exógena, y permitir el desarrollo de una inmunidad cruzada frente a diferentes aislados (Monney & Hemphill, 2014; Reichel & Ellis, 2009). Paralelamente, la vacunación del perro se ha presentado como una medida deseable debido a su papel en la transmisión horizontal y en la conexión con los ciclos silváticos de la enfermedad (Reichel *et al.*, 2014). No obstante, el número de estudios de vacunación que se han llevado a cabo en el perro es muy reducido (Nishikawa *et al.*, 2000a). En un sentido más amplio, toda formulación vacunal debe cumplir unos requisitos de seguridad que garanticen que el inóculo empleado no pueda revertir hacia la virulencia, lo cual es esencial en el caso de las vacunas vivas atenuadas. Además, desde un punto de vista comercial, la estabilidad de la vacuna en el tiempo es un factor clave de especial relevancia para las vacunas vivas. Por otro lado, y teniendo en cuenta que el diagnóstico serológico supone la base del control y seguimiento de la infección a nivel del rebaño, es importante la posibilidad de desarrollar vacunas DIVA (*Differentiating Infected from Vaccinated Animals*) que permitan diferenciar a los animales vacunados de los infectados naturalmente.

3.2.1. Vacunas vivas atenuadas

El éxito del empleo de vacunas vivas radica en que permiten un adecuado procesamiento y presentación del antígeno, de modo que el sistema inmunitario es activado de la misma manera que en el caso de una infección natural (Innes & Vermeulen, 2006). Dado que en la actualidad existe una vacuna viva comercial frente a la toxoplasmosis ovina, el desarrollo de un preparado de las mismas características frente a *N. caninum* parece factible. Dicha vacuna está basada en la cepa no persistente S48 (Toxovax™, Buxton, 1993), que es incapaz de conferir protección cruzada frente a la neosporosis bovina (Innes *et al.*, 2001a; Innes *et al.*, 2001b).

El empleo de vacunas vivas atenuadas ha mostrado los mejores resultados en términos de protección frente a la transmisión vertical. Éstas han sido evaluadas en el modelo murino (Rojo-Montejo *et al.*, 2012; Miller *et al.*, 2005) y en el bovino (Hecker *et al.*, 2013; Rojo-Montejo *et al.*, 2013; Weber *et al.*, 2012; Williams *et al.*, 2007). En concreto, destacan las vacunas basadas en los aislados Nc-Nowra (Miller *et al.*, 2002) y Nc-Spain1H (Rojo-Montejo *et al.*, 2009b), que fueron obtenidos a partir de animales asintomáticos con infección congénita. La administración de estas vacunas confirió elevados porcentajes de eficacia frente al aborto en las infecciones experimentales llevadas a cabo en el ganado bovino (Rojo-Montejo *et al.*, 2013; Weber *et al.*, 2012; Williams *et al.*, 2007), y frente a la transmisión vertical y la mortalidad neonatal en el modelo murino (Rojo-Montejo *et al.*, 2012; Ellis *et al.*, 2008; Miller *et al.*, 2005) (Tabla 2). No obstante, la seguridad de estos preparados sólo ha sido evaluada en el caso del aislado Nc-Spain1H, cuya administración no se asocia al desarrollo de reacciones adversas locales ni sistémicas en el ratón y en el ganado bovino (Rojo-Montejo *et al.*, 2013; Rojo-Montejo *et al.*, 2012).

Por otra parte, caben destacar diversos trabajos en los que la atenuación del parásito se ha realizado *in vitro* mediante pases sucesivos en cultivo celular (Bartley *et al.*, 2008; Bartley *et al.*, 2006), a través de la irradiación de los taquizoítos con rayos gamma (Ramamoorthy *et al.*, 2006) y generando parásitos mutantes sensibles a la temperatura (Lindsay *et al.*, 1999c) o capaces de expresar constitutivamente antígenos específicos del estadio de bradizoíto (Marugán-Hernández *et al.*, 2011b).

A pesar de los buenos resultados que se han obtenido hasta el momento, la comercialización de vacunas vivas cuenta con ciertos inconvenientes asociados a su alto coste de producción y su corta estabilidad a largo plazo (Reichel *et al.*, 2015). Por otro lado, el empleo de vacunas vivas suscita el debate de la posible reversión de la virulencia de aquellos aislados vacunales atenuados *in vitro*, tal y como se plantea con la vacuna comercial existente frente a la toxoplasmosis ovina (Buxton *et al.*, 1991). Por este motivo, existe un notable interés en el desarrollo de vacunas inactivadas o de subunidades que eviten estos inconvenientes.

Modelo animal	Vacuna	Desafío	Infección	Tasa de protección	Criterio de protección	Referencia
Murino cerebral	Nc-Spain1H $5x10^5$ s.c.	Nc-Liv $2x10^6$ s.c.	6-10 d.g.	89,5 %	Presencia del parásito en cerebro	(Rojo-Montejo *et al.*, 2012)
Murino gestante	Nc-Nowra $1x10^4$ s.c.	Nc-Liv $1x10^6$ s.c.	5 d.g.	89,5-98,8 %	Transmisión vertical	(Miller *et al.*, 2005)
	Nc-Spain1H $5x10^5$ s.c.	Nc-Liv $2x10^6$ s.c.	6-10 d.g.	85 %	Transmisión vertical	(Rojo-Montejo *et al.*, 2012)
Bovino gestante	Nc-Nowra $1x10^7$ i.v.	Nc-Liv 10^7 i.v.	70 d.g.	100 %	Muerte fetal y transmisión vertical	(Williams *et al.*, 2007)
	Nc-Spain1H 10^7 s.c	Nc-1 10^7 i.v.	70 d.g.	50 %	Muerte fetal	(Rojo-Montejo *et al.*, 2013)
	Nc-Spain1H 10^7 s.c	Nc-1 $4x10^8$ i.v.	135 d.g.	40%	Transmisión vertical	(Rojo-Montejo *et al.*, 2013)

Tabla 2. Eficacia de diferentes vacunas vivas basadas en aislados atenuados en los modelos murino y bovino. i.p.: intraperitoneal. s.c.: subcutáneo. i.v.: intravenoso. d.g.: días de gestación.

3.2.2. Vacunas inactivadas

Las vacunas inactivadas han sido probadas en el modelo murino (Liddell *et al.*, 1999) y en los modelos bovino (Choromanski & Block, 2000) y ovino (Jenkins *et al.*, 2004b; O'Handley *et al.*, 2003). Pese a ser más seguras que las vacunas vivas, confieren un menor grado de protección por estimular en menor medida la respuesta inmunitaria del hospedador, lo que se compensa con la administración de dosis sucesivas de recuerdo y con el empleo de adyuvantes (Babiuk, 2002). Estos preparados tienen una mayor estabilidad en el tiempo y un coste de producción y distribución relativamente más bajo que las vacunas vivas atenuadas, pero hasta el momento todas las formulaciones probadas han sido incapaces de prevenir la transmisión vertical del parásito (Innes *et al.*, 2011).

Este tipo de vacunas pueden estar formadas por el taquizoíto completo o por extractos antigénicos del mismo. En las primeras, la inactivación del parásito se consigue por métodos físicos, como el calor, o mediante diversos métodos químicos. Lamentablemente, ambos abordajes pueden producir alteraciones de la composición antigénica del parásito, lo que repercute negativamente en la capacidad inmunogénica de la vacuna. De entre todos los métodos químicos existentes, la etilamina binaria parece ser más respetuosa con la estructura de los antígenos del parásito. Este compuesto se ha utilizado en vacunas ensayadas en modelos murinos (Rojo-Montejo *et al.*, 2011a; Rojo-Montejo *et al.*, 2011b) y bovinos (Andrianarivo *et al.*, 2000). De hecho, esta última vacuna estuvo comercialmente disponible frente a la neosporosis bovina bajo el nombre de Neoguard®, si bien fue descatalogada recientemente por conferir una protección muy limitada (Weston *et al.*, 2012).

Por otro lado, las vacunas basadas en extractos del parásito comprenden una mezcla compleja de antígenos cuya composición puede variar en función de si el extracto es soluble, insoluble o total (Reichel & Ellis, 2009). Hasta el momento, la mayoría de los estudios se han llevado a cabo con extractos solubles, aunque su eficacia ha sido limitada (Mansilla *et al.*, 2013; Mansilla *et al.*, 2012; Miller *et al.*, 2005). La baja eficacia vacunal de dichos extractos podría deberse a que carecen de las proteínas de membrana, que son esenciales para la interacción inicial del parásito con la célula hospedadora (Hemphill *et al.*, 2013). Curiosamente, la vacunación con antígenos de excreción-secreción del taquizoíto se ha asociado a una exacerbación de los signos de la enfermedad tras el desafío, probablemente debido a su papel en la invasión de la célula hospedadora (Ribeiro *et al.*, 2009).

3.2.3. Vacunas de nueva generación

De forma similar al resto, en los últimos años el desarrollo de vacunas de subunidades basadas en ADN o proteínas recombinantes de expresión heteróloga ha sido destacable. Estas vacunas agrupan cuatro características esenciales: simplicidad de producción, mayor estabilidad en el tiempo, mayor seguridad y posibilidad de diferenciar entre animales infectados y vacunados (Reichel & Ellis, 2009). Las vacunas de subunidades serían capaces de bloquear procesos específicos en la biología del parásito al desencadenar una respuesta inmunitaria expresamente dirigida frente a dianas concretas. Por esta razón, las proteínas involucradas en el ciclo lítico del taquizoíto (superficie, micronemas, roptrias y gránulos densos), así como aquellas expresadas de forma específica durante el estadio de bradizoíto, han sido consideradas como posibles candidatos vacunales.

A pesar de las ventajas que conlleva su empleo, las vacunas recombinantes han demostrado una eficacia muy baja en comparación con las vacunas vivas, tanto en modelos murinos (Monney & Hemphill, 2014) como bovinos (Nishimura *et al.*, 2013). Sin embargo, ante la existencia de dos vacunas basadas en antígenos purificados y disponibles comercialmente frente a *Eimeria maxima* (CoxAbic™) y *Babesia* spp. (Novibac Piro™, actualmente descatalogada), el desarrollo de una vacuna recombinante frente a la neosporosis bovina parece factible. Diversos estudios han descrito un incremento de la eficacia de las formulaciones polivalentes frente a las monovalentes (Debache *et al.*, 2009; Cho *et al.*, 2005), por lo que las primeras constituyen una opción más deseable. Lamentablemente, uno de los problemas derivados del empleo de proteínas recombinantes como antígenos vacunales es la ausencia de reconocimiento del antígeno nativo por parte de los animales inmunizados, lo que conlleva un fracaso en la protección frente al desafío con el parásito como se ha reportado en estudios previos (Aguado-Martínez *et al.*, 2009a; Cannas *et al.*, 2003a). Además, desde un punto de vista práctico, la evaluación de dichas dianas debería llevarse a cabo en un modelo gestante, ya que éste se ajusta mejor al escenario natural de la neosporosis bovina. Los modelos gestantes permiten evaluar las consecuencias de la infección en la descendencia tras la vacunación y proporcionan el resto de los parámetros evaluables en un modelo cerebral, pero bajo la

influencia de la modulación inmunológica que se produce durante la gestación de forma fisiológica (Tabla 3).

En lo que respecta a las vacunas recombinantes probadas hasta la fecha, la proteína de superficie NcSRS2 ha conferido buenos resultados de protección en diversos estudios llevados a cabo en el modelo murino cerebral (Tuo *et al.*, 2011; Cannas *et al.*, 2003a). De la misma forma, los estudios realizados con epítopos de esta proteína en el ganado bovino muestran respuestas inmunitarias similares a las que se desarrollan tras una infección, lo que la convierte en una candidata interesante para prevenir el aborto (Baszler *et al.*, 2008). Por otro lado, la inmunización con la proteína de superficie NcSAG1 protegió frente a la infección cerebral en el modelo murino (Cannas *et al.*, 2003a), mientras que no confirió protección alguna frente a la transmisión vertical en el modelo bovino (Hecker *et al.*, 2014).

Estirpe de ratón	Vacuna	Desafío	Infección y sacrificio	Supervivencia en crías	Referencia
BALB/c	rNcROP2Fam-1, rNcMIC1, rNcMIC3 i.p.	Nc-1 $2x10^6$ i.p.	7-11 d.g. 50 d.p.p.	20 - 50 %	(Debache *et al.*, 2009)
	rNcGRA7, rNcSAG4 s.c.	Nc-1 $2x10^6$ s.c.	7-11 d.g. 30 d.p.p.	0 - 7,7 %	(Aguado-Martínez *et al.*, 2009a)
	rNcAMA1 s.c.	Nc-1 $1x10^5$ i.p.	8-10 d.g. 30 d.p.p.	57,7 - 62,5 %	(Zhang *et al.*, 2010)
	rNcGRA7, rNcSAG4, rNcBSR4, rNcSRS9 s.c.	Nc-Liv $2x10^6$ s.c.	7-11 d.g. 30 d.p.p.	0 %	(Jiménez-Ruiz *et al.*, 2012)
	rNcMIC3-MIC1-ROP2Fam-1 i.p.	Nc-1 $2x10^6$ i.p.	7-11 d.g. 34 d.p.p.	0 - 11,1 %	(Monney *et al.*, 2012; Monney *et al.*, 2011)
	rNcPDI i.n.	Nc-1 $2x10^6$ i.p.	7-11 d.g. 30 p.p.	14,3 - 19,1 %	(Debache & Hemphill, 2013)
C57BL/6	rNcMIC1, rNcMIC2, rNcGRA2, rNcGRA6, rNcSRS2 en *B. abortus* i.p.	Nc-1 $5x10^6$ i.p.	11-13 d.g. 21 d.p.p.	50 - 95,5 %	(Ramamoorthy *et al.*, 2007c)
Qs	rNcGRA1, rNcGRA2, rNcMIC10, rNcp24B s.c.	Nc-Liv 10^6 s.c.	5 d.g. 7 d.p.p.	58,5 - 100 %	(Ellis *et al.*, 2008)

Tabla 3. Eficacia de diferentes vacunas basadas en proteínas recombinantes en el modelo murino gestante. i.p.: intraperitoneal. s.c.: subcutáneo. i.n.: intranasal. d.g.: días de gestación. d.p.p.: días postparto.

Los ensayos de vacunación realizados con las proteínas de micronemas MIC han mostrado resultados moderados o nulos en el modelo murino cerebral (Srinivasan *et al.*, 2007; Alaeddine *et al.*, 2005; Cannas *et al.*, 2003b), aunque en algunos casos se ha observado una reducción significativa de la carga parasitaria tras el desafío en el mismo modelo (Monney *et al.*, 2011; Debache *et al.*, 2009; Alaeddine *et al.*,

2005). Además, la vacunación de ratones con la proteína de micronemas NcPDI se ha asociado a una disminución de la carga cerebral del parásito, mientras que no desencadena ningún efecto protector en el modelo gestante (Debache & Hemphill, 2013).

Hasta la fecha, la única proteína de roptrias empleada como vacuna es NcROP2Fam-1, cuyo empleo ha arrojado resultados esperanzadores tanto en el modelo murino cerebral como en el gestante. Las vacunas basadas en la proteína NcROP2Fam-1 destacan principalmente por su capacidad para reducir la mortalidad neonatal y la carga parasitaria en la descendencia (Debache *et al.*, 2010; Debache *et al.*, 2009; Debache *et al.*, 2008).

El uso de las proteínas de gránulo densos NcGRA1, NcGRA2, NcGRA6 y NcGRA7 como vacunas en el modelo murino cerebral y gestante se ha asociado a una protección y reducción de la carga parasitaria parciales (Ellis *et al.*, 2008; Cho *et al.*, 2005; Jenkins *et al.*, 2004a). En contraste, pese a estimular una fuerte respuesta inmunitaria de base humoral y celular, la protección conferida por NcGRA7 en otros estudios similares fue muy baja (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a). Del mismo modo, los experimentos llevados a cabo en el modelo bovino con NcGRA7 aportan resultados discordantes. Por un lado, la vacunación con esta proteína se ha asociado a una reducción de la carga parasitaria en el cerebro (Nishimura *et al.*, 2013). No obstante, su administración resulta incapaz de bloquear la transmisión transplacentaria (Hecker *et al.*, 2014).

Las proteínas específicas del estadio de bradizoíto también han constituido una importante vía de investigación. Sin embargo, la mayoría de los estudios describen una eficacia muy limitada tras la inmunización con NcSAG4, NcMAG1, NcSRS9 y NcBSR4 en ratones (Jiménez-Ruiz *et al.*, 2012; Debache *et al.*, 2010; Aguado-Martínez *et al.*, 2009a). En contraposición, la vacunación con las proteínas NcBAG1, NcMAG1 y NcSAG4 se ha asociado a una disminución de la carga parasitaria en el cerebro en el modelo murino (Uchida *et al.*, 2013).

El análisis de la respuesta inmunitaria desencadenada tras la vacunación y el desafío en los diferentes ensayos realizados con proteínas recombinantes hasta la fecha también arroja resultados dispares. Mientras ciertos autores asocian la protección frente al parásito con una respuesta del tipo Th1 (Nishikawa *et al.*, 2009), otros destacan que el papel de la respuesta Th2 tiene una mayor influencia (Haldorson *et al.*, 2005). No obstante, y pese a estas discrepancias, todos coinciden en la importancia del adyuvante elegido y en el sistema de expresión empleado para la obtención de la proteína recombinante (Rojo-Montejo *et al.*, 2011a; Debache *et al.*, 2010; Nishikawa *et al.*, 2009; Jenkins *et al.*, 2004a). Sin embargo, hay que destacar que la comparación directa entre estudios es limitada por la amplia heterogenicidad de modelos animales, adyuvantes y dosis vacunales, esquemas de vacunación y sistemas de expresión empleados en los diferentes trabajos publicados hasta la fecha.

Dentro del concepto de vacunas de nueva generación se incluyen también las vacunas de ADN. Hasta el momento se han realizado numerosos ensayos con este tipo de formulaciones codificando para las proteínas Nc*SRS2* (Cannas *et al.*, 2003a), Nc*MIC1* (Srinivasan *et al.*, 2007) y Nc*GRA7* (Jenkins *et al.*, 2004a; Liddell *et al.*, 2003), entre otros. Sin embargo, no existe consenso sobre si los resultados con este tipo de vacunas son mejores que los obtenidos con las proteínas recombinantes propiamente dichas (Srinivasan *et al.*, 2007; Alaeddine & Hemphill, 2004). Otras vacunas a destacar son las de vectores, que inducen una inmunidad de base humoral y celular, y facilitan la distinción entre los animales vacunados e infectados. Hasta la fecha, el virus Vaccinia-NcSRS2 ha sido uno de los vectores más empleados en el desarrollo de vacunas frente a *N. caninum* (Nishikawa *et al.*, 2001d; Nishikawa *et al.*, 2000a; Nishikawa *et al.*, 2000b), aunque *Brucella abortus* ha sido también utilizada como vector vacunal capaz de expresar de forma heteróloga las proteínas NcMIC1, NcMIC3, NcGRA2, NcGRA6 y NcSRS2 (Ramamoorthy *et al.*, 2007b; Ramamoorthy *et al.*, 2007c; Vemulapalli *et al.*, 2007).

3.3. Bases para el desarrollo de vacunas

3.3.1. Adyuvantes

El desarrollo de una nueva vacuna no puede ser concebido sin la elección de un adyuvante apropiado. Los adyuvantes son sustancias que potencian la respuesta inmunitaria desencadenada por el antígeno administrado, y su importancia es clave en vacunas que no sean vivas y que, por tanto, tienen mermada su capacidad para activar *per se* la respuesta del hospedador. La administración de una vacuna debería estimular al sistema inmunitario de forma análoga a como ocurriría tras una infección natural, esto es, activando inicialmente la respuesta innata para que ésta desemboque en una respuesta de tipo adaptativa con adquisición de memoria inmunológica. Este efecto sólo se consigue con una combinación adecuada de un extracto antigénico con uno o varios adyuvantes. Idealmente, un adyuvante debería promover el desarrollo de una respuesta inmunitaria con la menor cantidad de efectos adversos posibles. Desde un punto de vista veterinario, dichos efectos se traducen en una disminución del crecimiento, una alteración de los parámetros reproductivos, la producción de lesiones en el punto de inoculación que deprecien el valor de la canal, e incluso la presentación de signos clínicos variables que comprometan el bienestar animal. Del mismo modo, un adyuvante debe ser compatible con una administración sencilla y a un coste relativamente bajo. La elección del tipo adyuvante a administrar junto con un preparado vacunal depende de muchos factores, entre los que destacan la naturaleza del antígeno y la vía de administración, que a su vez determinará la magnitud, el tipo de respuesta inmunitaria y la duración de la misma (Heegaard *et al.*, 2011; Singh & O'Hagan, 2003).

Los adyuvantes más empleados hasta el momento incluyen las emulsiones y las sales de sales de fosfato o hidróxido de aluminio. Las emulsiones de aceite en agua son adyuvantes muy eficientes que

favorecen el desarrollo de una inmunidad de larga duración cuando son combinadas con proteínas recombinantes. No obstante, su mayor eficiencia se asocia a una mayor cantidad de efectos adversos, como la presentación de fiebre y la formación de granulomas en el punto de inoculación. Estos efectos son aún más llamativos cuando los aceites de la emulsión son de base mineral. Por el contrario, las emulsiones de aceite en agua favorecen el desarrollo de una inmunidad de corta duración, aunque son más seguras. Estos adyuvantes suelen ser empleados en el diseño de vacunas vivas y de ADN, y precisan de un mayor número de administraciones de recuerdo. Mientras que las emulsiones son capaces de inducir una respuesta inmunitaria de tipo Th1 y Th2, las sales de fosfato o hidróxido de aluminio, favorecen las respuestas de tipo Th2 (Heegaard *et al.*, 2011). El mecanismo de acción de ambos adyuvantes se basa en la adsorción del antígeno vacunal en un reservorio formado en el punto de inoculación que permite su liberación lenta, mantiene un estímulo inmunitario prolongado, e impide su rápida degradación. Por otro lado, las saponinas han sido compuestos tradicionalmente empleados como adyuvantes en diversas vacunas de uso veterinario. Estos compuestos están formados por mezclas complejas de glicósidos triterpenoides obtenidas de la corteza del árbol *Quillaja saponaria* que son capaces de estimular respuestas de tipo Th1 (Sun *et al.*, 2009). Más concretamente, el Quil-A es uno de los compuestos más utilizados que se obtiene tras una purificación parcial del extracto anterior. Este adyuvante es de especial interés por su aparente utilidad y eficacia en diversas vacunas diseñadas frente a parásitos apicomplejos (Bitencourt *et al.*, 2013; Igarashi *et al.*, 2010; García *et al.*, 2007).

Además de los adyuvantes clásicos, ciertas sustancias inmunoestimulantes pueden ser empleadas con el mismo fin por ser capaces de activar por sí mismas la respuesta inmunitaria innata. En los últimos tiempos han cobrado importancia los agonistas de los receptores TLR, que producen inflamación a nivel local, permiten el reclutamiento de células dendríticas en el punto de inoculación, y favorecen el desarrollo de memoria inmunológica. Un ejemplo de estos agonistas es el de los oligodesoxinucleótidos sintéticos CpG, que imitan los motivos CpG únicamente presentes en el genoma de microorganismos patógenos y activan los receptores TLR9, favoreciendo respuestas de tipo Th1 (Heegaard *et al.*, 2011). Por otro lado, los liposomas y las micro/nanopartículas son capaces de englobar al antígeno y de permitir su fagocitosis progresivamente por las células presentadoras de antígeno, potenciando el desarrollo de una respuesta apropiada. Además, estos compuestos parecen potenciar el desarrollo de la inmunidad de mucosas, del mismo modo que otros potenciadores como son el ácido retinoico, el interferón y la toxina colérica (Debache & Hemphill, 2013; Heegaard *et al.*, 2011; Debache *et al.*, 2010). En la actualidad, muchos de los adyuvantes empleados en el desarrollo de vacunas combinan dos o más características de las descritas previamente. El ejemplo es el adyuvante completo de Freund, que combina una emulsión de aceite mineral con extractos de micobacterias altamente inmunogénicos. También son importantes las partículas ISCOMs (*Immune Stimulating Complexes*), que engloban al antígeno para favorecer su fagocitosis, pero que además contienen Quil-A en su matriz (Heegaard *et al.*, 2011).

Hasta la fecha son muchos los compuestos que se han empleado en el diseño de vacunas contra la neosporosis bovina (Monney & Hemphill, 2014; Monney *et al.*, 2011; Reichel & Ellis, 2009). Sin embargo, son pocos los estudios en los que la eficacia de un mismo preparado antigénico se pruebe en combinación con diferentes adyuvantes. No obstante, la influencia del adyuvante en el éxito o fracaso de una misma mezcla antigénica ha quedado de manifiesto en trabajos previos (Rojo-Montejo *et al.*, 2011a; Andrianarivo *et al.*, 1999).

3.3.2. Modelos *in vivo*

El desarrollo de una vacuna frente a la neosporosis bovina parte de la idea del "*proof-of-concept*", o lo que es lo mismo, la comprobación de que un producto concreto pueda ser explotado de forma útil y rentable en el futuro. Las pruebas de concepto permiten determinar la eficacia y seguridad de los nuevos candidatos vacunales mediante su evaluación en modelos animales en los que la enfermedad se reproduce de forma experimental. Actualmente no existe un modelo animal estandarizado que pueda ser empleado de forma generalizada por todos los grupos de investigación centrados en el estudio de la neosporosis bovina, por lo que la comparación de los resultados obtenidos entre los mismos es difícil. Por este motivo, la armonización de modelos es una tarea urgente (Arranz-Solís *et al.*, 2015a; Benavides *et al.*, 2014).

3.3.2.1. Modelo murino

Por su reducido tamaño y coste, fácil manejo, corto periodo de gestación y mayor disponibilidad comercial de reactivos inmunológicos, los modelos murinos son los que se emplean con mayor frecuencia para la evaluación de vacunas frente a *N. caninum*. No obstante, por lo general esta especie presenta una susceptibilidad limitada a la infección. Desde la primera descripción de *N. caninum* por Bjerkås en 1984, el ratón ha sido utilizado para la caracterización del parásito en diferentes ámbitos. Estos incluyen la patogenia de la infección (López-Pérez *et al.*, 2006; Nishikawa *et al.*, 2001c; Eperon *et al.*, 1999), la respuesta inmunitaria (Ritter *et al.*, 2002; Khan *et al.*, 1997), la caracterización *in vivo* de diferentes aislados (Dellarupe *et al.*, 2014a; Pereira García-Melo *et al.*, 2010; Regidor-Cerrillo *et al.*, 2010a; Collantes-Fernández *et al.*, 2006c; Atkinson *et al.*, 1999; Stenlund *et al.*, 1997), y el desarrollo de vacunas frente a *N. caninum* (Monney & Hemphill, 2014; Monney *et al.*, 2011; Reichel & Ellis, 2009).

En lo que respecta al desarrollo de vacunas frente a la neosporosis bovina, el número de modelos murinos utilizados hasta la fecha es amplio y heterogéneo. En este sentido, es importante subrayar que las consecuencias de la infección experimental por *N. caninum* son muy variables y dependen de la estirpe de ratón, entre otros factores. Los ratones no consanguíneos de las estirpes Swiss Webster (Lindsay & Dubey, 1989), ICR (Lindsay *et al.*, 1995), CD-1 (Khan *et al.*, 1997) y Quanckenbush (Qs) (Miller *et al.*, 2002) son poco susceptibles a la infección si no se les co-administran fármacos inmunosupresores (Collantes-

Fernández *et al.*, 2004; Lindsay *et al.*, 1999c; Lindsay & Dubey, 1989). En contraposición, los ratones consanguíneos BALB/c y C57BL/6 muestran una mayor susceptibilidad a la infección de forma natural (Ramamoorthy *et al.*, 2007a; Lindsay *et al.*, 1995), aunque los primeros desarrollan signos clínicos más evidentes, lo que facilita la monitorización de la infección. De hecho, una importante cantidad de estudios se ha centrado únicamente en la estirpe BALB/c (Reichel & Ellis, 2009) (Figura 5).

Figura 5. Evolución del crecimiento de una camada no infectada en el modelo murino congénito de neosporosis bovina basado en la estirpe de ratón BALB/c.

Los modelos basados en los ratones BALB/c se pueden dividir en dos: el modelo murino cerebral, que se lleva a cabo con hembras no gestantes, y el modelo congénito, en el que se emplean hembras gestantes.

El modelo murino cerebral aporta información relativa a la fase aguda y crónica de la infección y a las consecuencias de la misma a nivel nervioso. Este modelo sería comparable a la transmisión horizontal en condiciones naturales y permite valorar la capacidad de una vacuna para conferir resistencia a la formación de quistes con bradizoítos mediante la valoración de los signos clínicos asociados a la infección. Durante la fase aguda, que se observa durante las primeras semanas, se produce una replicación activa del parásito en el hígado, bazo, linfonódulos y pulmón (Pereira García-Melo *et al.*, 2010; Collantes-Fernández *et al.*, 2006b). Esta fase se manifiesta con apatía, anorexia, erizamiento del pelo y disnea en los casos más graves. La fase crónica se instaura a partir de la segunda semana de la infección. En ella, el número de taquizoítos detectables en el pulmón comienza a descender de forma considerable y empiezan a detectarse en mayores proporciones en el cerebro (Collantes-Fernández *et al.*, 2006b). A partir de entonces aumenta la expresión de la proteína NcSAG4, considerada un marcador específico de la fase crónica de la enfermedad (Aguado-Martínez *et al.*, 2009b), y se empiezan a detectar niveles de IgG2a por encima de los de IgG1 (Aguado-Martínez *et al.*, 2009b; Collantes-Fernández *et al.*, 2006b). Aunque es poco frecuente, los animales pueden empezar a manifestar signos nerviosos tales como debilidad, paresia o parálisis, ataxia, síndrome vestibular con torneo e incluso la muerte (Reichel & Ellis, 2009; Long *et al.*, 1998). La valoración de la eficacia de una vacuna en este modelo suele fundamentarse en la cuantificación de las cargas parasitarias en los órganos diana del parásito (Collantes-Fernández *et al.*, 2006b).

Por su parte, el modelo murino congénito reproduce la transmisión transplacentaria exógena, por lo que determinaría la capacidad de una vacuna para bloquear la transmisión vertical del parásito tras una primo-infección, la aparición de signos clínicos y la multiplicación del parásito en los órganos diana. Este modelo permite evaluar la morbilidad y mortalidad neonatal, la tasa de transmisión vertical y el tiempo de supervivencia medio de la descendencia, y ha sido utilizado por diferentes grupos de investigación en los últimos años (Debache & Hemphill, 2013; Jiménez-Ruiz *et al.*, 2012; Monney *et al.*, 2012; Rojo-Montejo *et al.*, 2012; Marugán-Hernández *et al.*, 2011b; Aguado-Martínez *et al.*, 2009a). No obstante, y a pesar de que la transmisión transplacentaria endógena constituye el principal modo de transmisión de *N. caninum* en condiciones naturales, hasta el momento no se dispone de un modelo capaz de mimetizar este fenómeno, y la mayoría de los estudios han sido incapaces de conseguir madres crónicamente infectadas que transmitan el parásito a la descendencia (Jiménez-Ruiz *et al.*, 2013a; Omata *et al.*, 2004; Cole *et al.*, 1995), lo que sugiere que el ratón no es una especie apropiada para el desarrollo de este tipo de modelos.

Es importante subrayar que las consecuencias de la infección experimental por *N. caninum* en el modelo BALB/c son muy variables y dependen de la fase de la gestación, del aislado de desafío, de la dosis aplicada y de la vía de administración. En primer lugar, la tasa de transmisión vertical de la infección depende del aislado de desafío (Dellarupe *et al.*, 2014b; Regidor-Cerrillo *et al.*, 2010a) y de la fase de la gestación en la que se produzca la infección (López-Pérez *et al.*, 2011; López-Pérez *et al.*, 2010; López-Pérez *et al.*, 2008; López-Pérez *et al.*, 2006). En este sentido, los aislados de mayor virulencia tienen mayor capacidad para atravesar la placenta y multiplicarse en el feto (Tabla 4), aunque las tasas de transmisión vertical disminuyen progresivamente a medida que avanza la gestación (López-Pérez *et al.*, 2006; Omata *et al.*, 2004; Quinn *et al.*, 2002b; Liddell *et al.*, 1999). Por otro lado, parece obvio pensar que la dosis de desafío y la vía de administración jueguen un papel clave en la infección, puesto que determinan la distribución del parásito. Dicha distribución depende del número de taquizoítos inoculados en primera instancia y de la facilidad de éstos para alcanzar el torrente circulatorio y diseminarse intraorgánicamente (Regidor-Cerrillo *et al.*, 2010a). Estas variables han sido recientemente estudiadas con la intención de refinar el modelo, que se considera demasiado agresivo. De hecho, se ha propuesto una reducción significativa de la dosis de desafío (Arranz-Solís *et al.*, 2015a).

A pesar de ser capaz de reproducir la infección cerebral y congénita por *N. caninum* y de desarrollar una respuesta inmunitaria similar a la observada en el ganado bovino, el modelo murino presenta ciertas limitaciones que restringen la extrapolación de los datos obtenidos hacia la especie de destino. En primer lugar, destaca la existencia de estirpes de ratón naturalmente resistentes a la infección por *N. caninum*. Además, no hay que olvidar que ambas especies presentan una estructura placentaria diferente (hemocorial *vs*. sindesmocorial), y que el periodo gestacional de la ratona es catorce veces inferior al de la vaca, lo cual acelera la obtención de resultados de eficacia vacunal, pero omite ciertos mecanismos inmunológicos que podrían desencadenarse a largo plazo frente al parásito en el transcurso

de la gestación. Por esta razón, el interés por el modelo ovino ha ido emergiendo en los últimos años. No obstante, el modelo murino permite llevar a cabo un cribado inicial de dianas vacunales de gran utilidad para el desarrollo de preparados eficaces frente a la neosporosis bovina.

3.3.2.2. Modelo ovino

La oveja se encuentra dentro del espectro de hospedadores de *N. caninum* (Dubey y Schares, 2011). De hecho, las infecciones naturales por *N. caninum* en el ganado ovino están ganando importancia por su papel en la producción de abortos, que tradicionalmente habían sido asociados a infecciones por *T. gondii* (Gonzalez-Warleta *et al.*, 2014; Moreno *et al.*, 2012; Dubey & Lindsay, 1990). Por lo tanto, el modelo ovino se presenta como una buena alternativa al bovino en vistas al desarrollo de fármacos o vacunas frente a *N. caninum* debido a su menor tamaño y costes de mantenimiento, gestaciones más cortas y estructura placentaria similar. Al igual que ocurre con los modelos murinos, los estudios llevados a cabo con ovejas son poco comparables entre sí por la diversidad de diseños experimentales llevados a cabo. Hasta la fecha sólo un estudio ha comparado las consecuencias de la infección por el aislado virulento Nc-Spain7 en los tres tercios de la gestación de la oveja bajo un mismo diseño experimental. Este trabajo concluye que las repercusiones de la infección son similares en la vaca y en la oveja, aunque en la segunda parecen ser más graves. De hecho, y al contrario que en la vaca, la infección durante el segundo tercio de la gestación produjo el aborto de todas las ovejas del estudio. Además, la infección durante el último tercio de la gestación dio lugar al nacimiento de corderos con infección congénita, pero llamativamente débiles (Arranz-Solís *et al.*, 2015b). A pesar de estas diferencias, que podrían estar influenciadas por la dosis de desafío, el modelo ovino se presenta como una buena alternativa al bovino.

3.3.2.3. Modelo bovino

El ganado bovino representa la especie ideal para desarrollar un modelo de neosporosis. De hecho, y al igual que en el modelo murino, la diversidad biológica de *N. caninum* ha sido demostrada en el modelo bovino (Regidor-Cerrillo *et al.*, 2014; Caspe *et al.*, 2012; Rojo-Montejo *et al.*, 2009a). No obstante, su empleo supone mayores costes y dificultad de manejo, así como prolongados periodos de experimentación por la duración de su gestación. Hasta el momento se han descrito una gran variedad de modelos que difieren entre sí en la raza empleada, el aislado, estadio parasitario y dosis de desafío, la ruta de administración y el momento del desafío. Por lo tanto, la comparación directa entre estudios también es difícil. Pese a ello, los trabajos realizados hasta la fecha han permitido profundizar en la dinámica del parásito durante las infecciones experimentales y en los mecanismos inmunológicos que subyacen a la infección (Benavides *et al.*, 2014).

Por su facilidad de obtención *in vitro*, la mayoría de los trabajos realizados han empleado taquizoítos de diferentes aislados y a diferentes dosis para reproducir experimentalmente la enfermedad.

No obstante, ciertos autores han conseguido reproducir experimentalmente la neosporosis bovina mediante la administración oral de ooquistes esporulados, pese a que son difíciles de obtener (Gondim *et al.*, 2004c; McCann *et al.*, 2007; Trees *et al.*, 2002). Aunque la proporción de abortos descrita en los trabajos donde el desafío se realizó con ooquistes es significativamente menor que en aquellos donde se llevó a cabo mediante la inoculación parenteral de taquizoítos, ciertos factores como la viabilidad de los esporozoítos, la dosis administrada y el momento de la gestación en el que se produjo la infección limitan la comparación directa con los estudios llevados a cabo con taquizoítos. En este sentido, la fase de la gestación en la que se produce la infección es uno de los determinantes del modelo, ya que las infecciones experimentales realizadas con taquizoítos durante el primer tercio producen la muerte fetal o el aborto (Gibney *et al.*, 2008), mientras que aquéllas que se retrasan hasta el segundo y último tercio se manifiestan con el nacimiento de terneros congénitamente infectados, con o sin signos clínicos (Almería *et al.*, 2010). A su vez, la gravedad y distribución de las lesiones observadas en el feto varían en función de la fase de la gestación en la que haya adquirido la infección (Benavides *et al.*, 2012; Collantes-Fernández *et al.*, 2006c).

Desde un punto de vista global, el modelo bovino de mortalidad fetal ha sido el más empleado. Este modelo desafía con taquizoítos a las hembras en el día 70 de gestación e induce la muerte fetal o el aborto (Regidor-Cerrillo *et al.*, 2014; Macaldowie *et al.*, 2004; Williams *et al.*, 2003; Williams *et al.*, 2000). Sin embargo, todos los modelos descritos hasta la fecha sólo emulan la transmisión transplacentaria exógena, y ningún otro modelo ha podido reproducir el modo de transmisión endógena hasta el momento, tal y como ocurre con los modelos murinos. No obstante, es posible llevar a cabo ciertos estudios de campo en los que se recurre al empleo de animales procedentes de rebaños infectados crónicamente (Benavides *et al.*, 2014).

3.3.3. Modelos *in vitro*

En la actualidad la investigación científica se encuadra dentro de estrictas políticas de bienestar animal fomentadas por la Unión Europea y que pretenden disminuir al máximo el empleo de animales de laboratorio. En este sentido, el desarrollo de modelos *in vitro* se ha presentado como una herramienta fundamental para el estudio de medidas de control frente a la infección por *N. caninum* (Müller & Hemphill, 2012). Estos modelos han permitido evaluar la eficacia de número importante fármacos diseñados frente al parásito, evitando el uso de modelos animales en aquellos compuestos que no tuvieran efecto significativo en sistemas *in vitro* (Ojo *et al.*, 2014; Barna *et al.*, 2013; Seo *et al.*, 2013; Debache & Hemphill, 2012; Mazuz *et al.*, 2012; Schorer *et al.*, 2012; Debache *et al.*, 2011a).

En el campo específico de las vacunas, los modelos *in vitro* han permitido identificar ciertos componentes del taquizoíto implicados en la interacción con la célula hospedadora (Aguado-Martínez *et al.*, 2008; Hemphill *et al.*, 1996), así como otros específicos del estadio de bradizoíto (Risco-Castillo *et al.*, 2011; Marugán-Hernández *et al.*, 2010; Risco-Castillo *et al.*, 2007; Vonlaufen *et al.*, 2004), ambos con interés

potencial como dianas vacunales. Por otro lado, los experimentos de proliferación linfocitaria resultan de gran utilidad para evaluar la respuesta inmunitaria de base celular desencadenada durante la fase inductora de un ensayo vacunal, siendo posible su determinación a través de una muestra de sangre (Regidor-Cerrillo *et al.*, 2014).

Hasta la fecha no se dispone de ningún modelo *in vitro* que sea lo suficientemente potente como para sustituir al modelo animal. No obstante, los abordajes *in vitro* son de gran utilidad cuando se utilizan como herramientas de cribado inicial para el desarrollo de dianas vacunales.

4. Variabilidad intra-específica de *N. caninum*

Hasta la fecha se han obtenido más de 90 aislados de *N. caninum* procedentes de animales domésticos (vaca, búfalo de agua, oveja y perro) y silvestres (ciervo de cola blanca, bisonte europeo y lobo) (Donahoe *et al.*, 2015; Dubey & Schares, 2011; Dubey *et al.*, 2007a). La disponibilidad de estos aislados y su comparación han facilitado el estudio de la variabilidad intra-específica del parásito, que se ha abordado tanto *in vivo*, por medio de modelos murinos y bovinos, como *in vitro*, mediante técnicas de cultivo celular. La variabilidad de *N. caninum* podría tener implicaciones negativas de cara al desarrollo de una vacuna, puesto que podría darse el caso de que un mismo preparado inmunológico no fuera capaz de proteger frente a otros aislados heterólogos presentes en la naturaleza. Por el contrario, el estudio comparativo de aquellos aislados con comportamientos biológicos diferentes permitiría la identificación de los factores que determinan la virulencia o avirulencia de los mismos. En este sentido, los abordajes moleculares se presentan como herramientas de gran utilidad para el estudio de la variabilidad intra-específica del parásito.

4.1. Variabilidad genética de *N. caninum*

La obtención de aislados de *N. caninum* procedentes de hospedadores y localizaciones geográficas muy diversas sugiere una elevada capacidad del parásito para adaptarse a diferentes nichos ecológicos. Esta capacidad podría llevar implícita una elevada diversidad genética que sería potenciada por la capacidad de recombinación y reorganización del material genético tras la reproducción sexual de individuos parentales diferentes, tal y como se ha descrito previamente en *T. gondii* (Grigg *et al.*, 2001).

Los primeros estudios de variabilidad genética entre aislados de *N. caninum* se fundamentaron en el análisis del ARNr 18S, así como de la región ITS-1 (*Internal Transcribed Spacer-1*) y de los RAPD (*Randomly Amplified Polymorphic DNA*). Debido a la elevada conservación de las secuencias de los marcadores analizados, estos abordajes indicaban una menor variabilidad genética que la descrita para *T. gondii* (Beck *et al.*, 2009). Posteriormente, el análisis de secuencias de microsatélites permitió evidenciar una elevada diversidad genética entre los aislados del parásito obtenidos *in vitro* (Pedraza-Díaz *et al.*, 2009; Regidor-Cerrillo *et al.*, 2006) y a partir de muestras de animales con infección natural (Basso *et al.*, 2010; Basso *et al.*, 2009; Regidor-Cerrillo *et al.*, 2008). De hecho, y gracias al análisis multilocus, ha sido posible definir una estructuración parcial de *N. caninum* en la que ciertos perfiles genéticos de cada aislado se han asociado a su país de origen (Regidor-Cerrillo *et al.*, 2013). No obstante, no parece existir vinculación entre la detección de determinados marcadores microsatélites y la virulencia de los aislados de *N. caninum*.

En contraposición, la estructura poblacional de *T. gondii* se encuentra completamente definida, habiéndose establecido hasta 15 haplogrupos que incluyen a las cepas clásicas de tipo I, II y III. De hecho,

estas últimas presentan SNPs (*Single-Nucleotide Polymorphism*) que afectan a los genes TgROP5, TgROP16 y TgROP18, y determinan su virulencia (Behnke *et al.*, 2011; Reese & Boothroyd, 2011; Saeij *et al.*, 2006; Taylor *et al.*, 2006). La utilidad de los SNPs como marcadores genéticos es mayor que la de los microsatélites, ya que presentan una menor tasa de mutación y se distribuyen homogéneamente por el genoma. En *N. caninum* la identificación de SNPs se ha llevado a cabo recientemente mediante la secuenciación de diferentes aislados. Los análisis llevados a cabo muestran que todos los genomas son ampliamente idénticos, aunque presentan pequeñas variaciones estructurales. Estos resultados sugieren que la población actual de *N. caninum* se debe a la expansión de un único linaje clonal que se ha visto favorecida por la transmisión vertical del parásito mediante reproducción asexual. No obstante, se han detectado importantes incongruencias entre la información del ADN de la mitocondria y el apicoplasto con la del ADN del núcleo. Estas discrepancias evidencian un proceso de recombinación sexual en el hospedador definitivo que explicaría la variabilidad intra-específica de los distintos aislados del parásito (Khan *et al.*, 2015).

4.2. Comportamiento *in vivo* e *in vitro* de los aislados de *N. caninum*

La heterogeneidad de los aislados de *N. caninum* se demostró por primera vez en modelos murinos. Estos estudios señalaron que las consecuencias de la infección por el parásito eran variables en función del aislado empleado, tanto en las madres como en la descendencia (Collantes-Fernández *et al.*, 2006b; Quinn *et al.*, 2002b; Atkinson *et al.*, 1999). Curiosamente, estos hallazgos tienen correlación con lo observado en los modelos bovinos (Gibney *et al.*, 2008; Williams *et al.*, 2000), así como con otros resultados obtenidos *in vitro* mediante técnicas de cultivo celular (Pérez-Zaballos *et al.*, 2005; Schock *et al.*, 2001). Todos estos trabajos tienen en común el origen de los aislados, que en su mayoría proceden de casos graves de la enfermedad. Por ejemplo, los aislados Nc-1 y Nc-Liv fueron obtenidos del encéfalo de perros con signos clínicos nerviosos manifiestos, mientras que el aislado Nc-SweB1 se obtuvo del encéfalo de un feto bovino abortado (Stenlund *et al.*, 1997; Barber *et al.*, 1995; Dubey *et al.*, 1988).

La obtención de aislados de *N. caninum* a partir de los casos clínicos más graves podría desviar el estudio del parásito sólo a aquéllos de mayor virulencia presentes en la población. En este sentido, los esfuerzos de diversos trabajos previos se centraron en la obtención de aislados de animales sanos pero congénitamente infectados y en la estandarización de modelos *in vivo* e *in vitro* para valorar las diferencias de comportamiento entre los aislados (Rojo-Montejo *et al.*, 2009b; Regidor-Cerrillo *et al.*, 2008). Los modelos murinos gestante y no gestante han sido los más utilizados para llevar a cabo la caracterización patogénica de los aislados en el ratón, si bien los primeros son los más adecuados porque reproducen la transmisión transplacentaria del parásito. Por su parte, la capacidad de invasión y proliferación de los taquizoítos puede ser determinada *in vitro*, aportando una idea de su actividad lítica sobre las células hospedadoras.

Al igual que con los aislados Nc-1, Nc-Liv y Nc-SweB1, la heterogeneidad de los aislados de *N. caninum* obtenidos en los últimos años ha sido demostrada *in vivo* (Dellarupe *et al.*, 2014a; Pereira García-Melo *et al.*, 2010; Regidor-Cerrillo *et al.*, 2010a; Rojo-Montejo *et al.*, 2009b) e *in vitro* (Dellarupe *et al.*, 2014b; Regidor-Cerrillo *et al.*, 2011; Rojo-Montejo *et al.*, 2009b). Además, se ha establecido una elevada correlación entre los resultados obtenidos mediante ambas aproximaciones (Regidor-Cerrillo *et al.*, 2011), e incluso entre las observaciones descritas en el modelo murino y en el modelo bovino, al menos con los aislados Nc-1, Nc-Spain1H y Nc-Spain7 (Regidor-Cerrillo *et al.*, 2014; Caspe *et al.*, 2012; Rojo-Montejo *et al.*, 2009a). En conjunto, estos trabajos han permitido clasificar a los aislados en función de su grado de virulencia (Tabla 4). Curiosamente, los aislados obtenidos a partir de ooquistes han mostrado una virulencia menor que aquellos de origen bovino (Dellarupe *et al.*, 2014a; Dellarupe *et al.*, 2014b).

La clasificación de los aislados de *N. caninum* en base a su virulencia abre nuevas vías de investigación en el campo de la patogénesis de la enfermedad, puesto que su comparación permitiría identificar posibles factores de virulencia cuyo conocimiento resulta esencial para el desarrollo de herramientas farmacológicas o inmunoprofilácticas que fueran capaces de bloquear procesos esenciales para el parásito.

Aislado	Origen, especie	Modelo murino gestante					Modelo *in vitro*		Consenso virulencia
		Morbilidad madres	Presencia parásito madres	Morbilidad neonatal	Mortalidad neonatal	Transmisión vertical	TI_{4h} [a]	PT_{56h} [b]	
Nc-6Arg	Heces, perro	Baja	Alta	Baja	Baja	Baja	Moderada	Moderada	Baja-moderada
Nc-Bahia *	Cerebro, perro	Alta	Alta	Alta	Alta	Alta	Alta	Alta	Alta
Nc-Ger2	Heces, perro	Baja	Baja	Baja	Baja	Baja	Baja	Baja	Baja
Nc-Ger3	Heces, perro	Baja	Moderada	Moderada	Moderada	Baja	Moderada	Alta	Moderada
Nc-Ger6	Heces, perro	Baja	Baja	Baja	Baja	Baja	Baja	Baja	Baja
Nc-Liv *	Cerebro, perro	Alta	Alta	Alta	Alta	Alta	Alta	Alta	Alta
Nc-Spain1H	Cerebro, ternero	Baja	Baja	Baja	Baja	Baja	Baja	Baja	Baja
Nc-Spain2H	Cerebro, ternero	Baja	Baja	Moderada	Moderada	Baja	Moderada	Baja	Baja-moderada
Nc-Spain3H	Cerebro, ternero	Baja	Moderada	Baja	Baja	Alta	Baja	Baja	Baja-moderada
Nc-Spain4H	Cerebro, ternero	Alta	Alta	Alta	Alta	Alta	Alta	Alta	Alta
Nc-Spain5H	Cerebro, ternero	Alta	Alta	Alta	Alta	Alta	Moderada	Alta	Alta
Nc-Spain6	Cerebro, ternero	Baja	Baja	Moderada	Moderada	Baja	Moderada	Alta	Baja-moderada
Nc-Spain7	Cerebro, ternero	Alta	Moderada	Alta	Alta	Alta	Moderada	Alta	Alta
Nc-Spain8	Cerebro, ternero	Baja	Baja	Baja	Baja	Baja	Moderada	Baja	Baja-moderada
Nc-Spain9	Cerebro, ternero	Baja	Baja	Moderada	Moderada	Baja	Moderada	Moderada	Baja-moderada
Nc-Spain10 *	Cerebro, ternero	Baja	Moderada	Moderada	Moderada	Moderada	Moderada	Alta	Moderada

Tabla 4. Clasificación de la virulencia de diferentes aislados de *N. caninum* en base a su comportamiento *in vivo* en modelos murinos gestantes e *in vitro* (Dellarupe *et al.*, 2014a; Dellarupe *et al.*, 2014b; Jiménez-Ruiz *et al.*, 2013b; Regidor-Cerrillo *et al.*, 2011; Pereira García-Melo *et al.*, 2010; Regidor-Cerrillo *et al.*, 2010a; Rojo-Montejo *et al.*, 2009b). * Aislados obtenidos de animales con signos nerviosos. N.D. No determinado. [a] Tasa de invasión a las 4 horas. [b] Proliferación de taquizoítos a las 56 horas.

4.3. Las técnicas -*omicas* en *N. caninum* y su aplicación para la identificación de nuevos candidatos vacunales

Las técnicas -*ómicas* aportan una visión global de los elementos implicados en los procesos biológicos a nivel molecular. Estas técnicas resultan de gran utilidad en el estudio de la variabilidad intra-especifíca de *N. caninum*, ya que permiten descifrar los mecanismos que subyacen a los rasgos fenotípicos de los diferentes aislados del parásito y determinar los procesos que determinan su virulencia. El auge de las técnicas –*ómicas* surgió tras la secuenciación y publicación del genoma de diversos patógenos eucariotas de importancia en salud pública y sanidad animal en la base de datos EuPathDB (*Eukaryotic Pathogen Database*, www.eupathdb.org) (Aurrecoechea *et al.*, 2010), que recoge información genómica, proteómica y transcriptómica de diversos géneros de parásitos apicomplejos como *Cryptosporidium*, *Plasmodium*, *Eimeria*, *Hammondia*, *Toxoplasma* y *Neospora*. Concretamente, la base de datos ToxoDB (www.toxodb.org) agrupa de forma libre y colaborativa la información -*ómica* disponible de estos últimos cuatro géneros (Gajria *et al.*, 2008).

4.3.1. Estudios genómicos

La genómica comprende la caracterización molecular de los genomas completos mediante el estudio de su contenido, organización y función de sus genes y la evolución de su información genética. En concreto, el genoma del aislado Nc-Liv, el único secuenciado y publicado hasta la fecha, cuenta con 61 Mb, 14 cromosomas y más de 7000 genes codificantes (Reid *et al.*, 2012).

Los primeros estudios genómicos a gran escala se basaron en los análisis de ESTs (*Expressed Sequence Tag*) (Li *et al.*, 2003; Ng *et al.*, 2002; Howe, 2001; Ajioka, 1998). Sin embargo, el avance de la tecnología en el campo de la genómica hizo posible la secuenciación completa del genoma de *T. gondii* y de *N. caninum*. No obstante, la información disponible para *T. gondii* es mucho mayor y comprende distintos aislados (ME49, GT1, VEG y RH). Curiosamente, el grado de homología del genoma de ambos parásitos ronda el 90%, por lo que tradicionalmente se ha utilizado a *T. gondii* como modelo para estudios de *N. caninum*. De hecho, se ha demostrado que los genomas de ambos parásitos son ampliamente sinténicos y que han sufrido una escasa ganancia o pérdida neta de genes debidas a los procesos de especiación (Reid *et al.*, 2012). Por otro lado, la comparación del genoma de distintos géneros de apicomplejos ha definido la existencia de familias de genes únicas que podrían estar asociadas a estrategias de evasión del sistema inmunitario por su elevada tasa de mutación (Reid, 2015). En el ámbito específico de *T. gondii*, dichas familias incluyen las proteínas de superficie SRS (*SAG1-Related Sequence*) (Reid *et al.*, 2012; Tomavo, 2001) y las quinasas presentes en las roptrias (ROPK) (Talevich & Kannan, 2013).

Por su parte, dado que el apicoplasto cuenta con su propio material genético, se encuentra en el punto de mira de muchos estudios en busca de nuevas dianas terapéuticas. Esta organela, que cuenta

todavía con su propia maquinaria de expresión génica, parece ser esencial para el parásito. Por este motivo su genoma ha sido completamente secuenciado en *T. gondii*, *N. caninum* y algunas especies del género *Plasmodium*.

4.3.2. Estudios transcriptómicos

La transcriptómica se encarga del estudio de los genes expresados en una etapa específica del desarrollo de una célula mediante el análisis de su ARN transcrito. La información obtenida de los estudios de transcriptómica sirve de nexo entre los hallazgos genómicos y proteómicos, aunque no tiene en cuenta los niveles de regulación existentes entre la transcripción y la traducción, como son la maduración del ARN y su transporte al citoplasma.

Los primeros análisis del transcriptoma del taquizoíto de *T. gondii* se realizaron mediante técnicas SAGE (*Serial Analysis Gene Expression*), ESTs, microarrays, MPSS (*Massively Parallel Signature Sequencing*) y TSSs (*Transcription Start Site*) (Hassan *et al.*, 2012; Bahl *et al.*, 2010; Yamagishi *et al.*, 2010; Wastling *et al.*, 2009; Radke *et al.*, 2005). No obstante, muchos de los estudios de transcriptómica llevados a cabo hasta la fecha se han centrado en la determinación de los mecanismos de manipulación de las células hospedadoras tras la infección. En concreto, se ha descrito que las proteínas TgROP16, TgGRA7 y TgGRA15 alteran ciertas rutas de transcripción tras la infección. Específicamente, la proteína TgROP16 es capaz de inducir cambios en la expresión de los factores de transcripción STAT3, 5 y 6 (Jensen *et al.*, 2013; Ong *et al.*, 2010; Yamamoto *et al.*, 2009), la proteína TgGRA7 puede modificar la expresión del gen p53 (Bougdour *et al.*, 2014), y la proteína TgGRA15 participa en la regulación la actividad del NF-κB (Rosowski *et al.*, 2011). Curiosamente, muchos de estos factores presentan importantes polimorfismos entre los distintos aislados de *T. gondii*, que condicionan ciertas características de la infección (Behnke *et al.*, 2011; Reese & Boothroyd, 2011; Saeij *et al.*, 2006; Taylor *et al.*, 2006). Por otro lado, y aunque no se conoce qué factores intervienen en ello, se ha descrito un aumento en la transcripción de micro-ARNs y del gen c-Myc en las células hospedadoras, cuya función comprende la regulación de la transcripción, la progresión del ciclo celular y la apoptosis (Franco *et al.*, 2014; Zeiner & Boothroyd, 2010). Además, los estudios transcriptómicos llevados a cabo *in vitro* (Okomo-Adhiambo *et al.*, 2006; Chaussabel *et al.*, 2003; Gail *et al.*, 2001) e *in vivo* (Pittman *et al.*, 2014; Jia *et al.*, 2013; Tanaka *et al.*, 2013; Skariah *et al.*, 2010) sobre células infectadas con *T. gondii* revelan que la infección por el parásito aumenta la expresión de genes relacionados con la respuesta inmunitaria y que esta expresión varía según el aislado empleado.

Al contrario de lo que ocurre con *T. gondii*, existen pocos estudios del transcriptoma de *N. caninum*. Recientemente, la expresión de genes de *T. gondii* y *N. caninum* se ha comparado a gran escala (Reid *et al.*, 2012). Este trabajo concluyó que el contenido genético de ambos parásitos está muy conservado, sobre todo en aquellos genes que regulan el metabolismo. Sin embargo, *N. caninum* mostró una expansión de los genes que codifican las proteínas de superficie SRS, así como una menor o

inexistente transcripción de factores de virulencia implicados en mecanismos de patogenicidad de *T. gondii* (NcROP18, NcROP16, NcROP5, NcSUB2). Por otro lado y al contrario que *T. gondii*, se ha demostrado recientemente que *N. caninum* es incapaz de aumentar la transcripción de micro-ARNs y del gen c-Myc, implicados en la regulación de la transcripción de la célula hospedadora y en el control de la progresión del ciclo celular y de la apoptosis (Franco *et al.*, 2014; Zeiner & Boothroyd, 2010). Lamentablemente, hasta el momento no se ha llevado a cabo ningún estudio que aporte información sobre los mecanismos de interacción del parásito con la célula hospedadora, ni que compare los niveles de expresión génica entre aislados con diferentes grados de virulencia.

4.3.3. Estudios proteómicos

La proteómica comprende el análisis global de las proteínas expresadas por un genoma en un momento determinado. Del mismo modo que se asume que *N. caninum* cuenta con una importante diversidad genética, es esperable que ésta tenga un reflejo directo en el proteoma de cada aislado. No obstante, si bien la exactitud de un estudio proteómico está directamente ligada a la calidad de la información genómica disponible, la integración de los datos proteómicos y genómicos permite el refinamiento de estos últimos (Krishna *et al.*, 2015; Wastling *et al.*, 2009; Xia *et al.*, 2008).

Durante los últimos años los abordajes proteómicos han surgido como herramientas muy útiles para el estudio de *N. caninum*. La composición proteica de los taquizoítos fue definida por primera vez mediante técnicas de electroforesis bidimensional (Lee *et al.*, 2004; Lee *et al.*, 2003). Esta misma metodología fue posteriormente empleada para determinar las diferencias en el proteoma de diferentes aislados del parásito (Shin *et al.*, 2005b), e incluso para comparar el proteoma de *N. caninum* y *T. gondii* (Zhang *et al.*, 2011; Lee *et al.*, 2005). Sin embargo, la electroforesis bidimensional aporta información meramente descriptiva, en contraste con la técnica de 2D-DIGE (*Two Dimensional-DIfference Gel Electrophoresis*), que permite cuantificar las diferencias en abundancia de aquellas proteínas diferencialmente expresadas entre dos extractos diferentes. La metodología de 2D-DIGE ha sido empleada para comparar el proteoma de las fases de taquizoíto y bradizoíto, revelando que los bradizoítos sufren un proceso de diferenciación hacía rutas glicolíticas anaeróbicas y una mayor expresión de proteínas de respuesta al estrés (*Heat Shock Proteins*) (Marugán-Hernández *et al.*, 2010).

Los estudios proteómicos llevados a cabo en *T. gondii* han ido orientados a la determinación de los factores de virulencia del parásito mediante la comparación del proteoma de aislados con diferentes grados de virulencia (Zhou *et al.*, 2014; Zhou *et al.*, 2013). Sin embargo, hasta el momento no se ha llevado a cabo ningún trabajo en el que se hayan determinado las diferencias existentes entre el proteoma de los taquizoítos y el de los bradizoítos. No obstante, y al contrario que en *N. caninum*, sí que se ha estudiado el proceso de modulación de las células infectadas en beneficio del parásito mediante el análisis de su proteoma antes y durante la infección (Nelson *et al.*, 2008; Zhou *et al.*, 2004). Estos últimos trabajos

concluyen que *T. gondii* es capaz de alterar la expresión de ciertas proteínas estructurales y metabólicas, así como otras relacionadas con la respuesta inmunitaria del hospedador y con el control de la apoptosis.

Los estudios del proteoma llevados a cabo con *N. caninum* han permitido la identificación de proteínas específicas de organelas de secreción (Marugán-Hernández *et al.*, 2011a; Sohn *et al.*, 2011), así como de los antígenos que estimulan las células T CD4+ bovinas (Rocchi *et al.*, 2011) y de las proteínas secretadas al inducir artificialmente la liberación de los depósitos de calcio intracelular (Pollo-Oliveira *et al.*, 2013). Por el contrario, en *T. gondii* se ha definido la composición proteica de la membrana plasmática (Chew *et al.*, 2012), de las roptrias (Bradley *et al.*, 2005), del citoesqueleto (Gómez de Leon *et al.*, 2014; Xia *et al.*, 2008), de las histonas (Nardelli *et al.*, 2013), e incluso de los ooquistes (Possenti *et al.*, 2013; Fritz *et al.*, 2012).

El estudio de la diversidad antigénica entre aislados de *N. caninum* de diferentes procedencias geográficas ha sido abordado mediante Western-blot en una (Shin *et al.*, 2005a; Miller *et al.*, 2002; Schock *et al.*, 2001) y dos dimensiones (Lee *et al.*, 2005; Shin *et al.*, 2005b), aunque la interpretación de las diferencias observadas en su inmunoma fue limitada. Pese a todo, no existen evidencias del desarrollo de una respuesta inmunitaria específica para cada aislado. De hecho, tal y como se ha demostrado en estudios previos de vacunación frente al parásito, es posible que la respuesta inmunitaria suscitada por un aislado reaccionara de forma cruzada frente a otro diferente, mostrando un patrón antigénico idéntico potencialmente deseable de cara al desarrollo de vacunas frente a la enfermedad (Williams *et al.*, 2007; Miller *et al.*, 2005; Jenkins *et al.*, 2004b).

JUSTIFICATION AND OBJECTIVES

Neospora caninum has emerged as the major cause of abortion in dairy and beef cattle worldwide, where it is responsible for losses in excess of a billion dollars annually (Reichel *et al.*, 2013). The parasite is considered one of the most efficiently transplacentally transmitted microbes in cattle. Thus, the infection can be maintained over several generations by vertical transmission in the affected herds (Dubey & Schares, 2011). Despite vaccination having proven to be an economically viable strategy for controlling the disease, vaccines or drugs against bovine neosporosis are not yet available (Reichel *et al.*, 2013), and control options are reduced to interrupting the parasite's life cycle through management measures addressed by proper diagnostic tools (Reichel *et al.*, 2014).

The negative economic impact of bovine neosporosis has turned the development of vaccines against the disease into an urgent need. Live vaccines have demonstrated high efficacy against the exogenous transplacental infection, but they have several disadvantages such as the potential for reversion to pathogenicity, costly production and distribution channels, and latency in the intermediate host (Reichel *et al.*, 2015). In this sense, inactivated and subunit vaccines appear as a good alternative to the use of live vaccines due to their reduced costs and the possibility to design them as DIVA formulations (Monney & Hemphill, 2014). Nevertheless, the former are composed of a heterogeneous mixture of parasite antigens, while the latter contain standardized amounts of specific peptides.

One of the most intriguing facts about the *N. caninum* biology is the intra-specific diversity displayed among isolates, which has been demonstrated both *in vitro* and *in vivo* in murine and bovine models (Dellarupe *et al.*, 2014a; Dellarupe *et al.*, 2014b; Regidor-Cerrillo *et al.*, 2011; Pereira García-Melo *et al.*, 2010; Regidor-Cerrillo *et al.*, 2010a; Rojo-Montejo *et al.*, 2009b). All the *N. caninum* isolates characterized to date display obvious differences in their biological behaviour, which suggest that those highly virulent are able to express components that allow them to invade and replicate more efficiently within the host cell. Similarly, this intra-specific diversity has been also defined in *T. gondii*, where three clearly defined clonal lineages and other atypical strains exhibiting differences in their virulence have been described (Xiao & Yolken, 2015). In *T. gondii*, this diversity has been exploited to determine those strain-specific components solely present in virulent strains and potentially related to virulence mechanisms (Adomako-Ankomah *et al.*, 2014; Reese & Boothroyd, 2011).

To date, most of the virulence factors described in *T. gondii* are clustered in the micronemes, rhoptries and dense granules (Mercier & Cesbron-Delauw, 2015; Etheridge *et al.*, 2014; Boothroyd & Dubremetz, 2008). These factors are sequentially released by the parasite during the lytic cycle and interact with the host cell to enable attachment, invasion, proliferation and egress with dramatic consequences for the infected tissues. In contrast, the knowledge about these factors in *N. caninum* is limited despite the fact that they also determine the harmful effects of the infection. Thus, proteins involved in the lytic cycle are ideal targets for the development of subunit vaccines against both toxoplasmosis and neosporosis (Hemphill *et al.*, 2013). Theoretically, vaccinated hosts would be able to

block those components involved in the host-parasite interactions, hampering the attachment and invasion processes (Hemphill *et al.*, 2013). However, and given the complexity of apicomplexan parasites that have acquired a number of mechanisms to evade the host immune responses, the development of polyvalent vaccines containing a variable number of parasite targets seems necessary (Monney & Hemphill, 2014). In *N. caninum* only six microneme proteins, two dense granule proteins and one rhoptry protein have been associated with the attachment and invasion processes during the lytic cycle (Hemphill *et al.*, 2013), and none of these components have shown to be involved in the hijack of the host cell machinery as described for *T. gondii* (Mercier & Cesbron-Delauw, 2015; Nolan *et al.*, 2015; Etheridge *et al.*, 2014). Therefore, substantial efforts should be made to increase the limited knowledge about the virulence factors of *N. caninum* in order to develop new control tools against the disease.

Due to their participation in the tachyzoites lytic cycle, the NcROP2Fam-1 and NcGRA7 proteins have been extensively studied in the last years. NcROP2Fam-1 is the only rhoptry protein characterized in *N. caninum*, where it has been associated with the invasion process (Alaeddine *et al.*, 2013). This protein belongs to the ROP2 family of kinases, which includes the major virulence factors described in *T. gondii* (Boothroyd, 2013; Boothroyd & Dubremetz, 2008; El Hajj *et al.*, 2006). In addition, the protein has been tested as a vaccine candidate in mouse models of neosporosis with high efficacy rates (Monney *et al.*, 2011; Debache *et al.*, 2010; Debache *et al.*, 2009; Debache *et al.*, 2008). On the other hand, NcGRA7 has been described as an immunodominant antigen (Álvarez-García *et al.*, 2007) involved in the parasite invasion and parasitophorous vacuole maturation (Aguado-Martínez *et al.*, 2010; Hemphill *et al.*, 1998). This protein has also been used as a vaccine candidate, showing promising results in terms of efficacy (Nishikawa *et al.*, 2009; Jenkins *et al.*, 2004a; Lally *et al.*, 1997). Based on this, the administration of NcROP2Fam-1 or NcGRA7 in combination with new vaccine candidates could presumably increase the number of essential processes affected in the parasite, thus improving the efficacy of the final formulation.

In the light of these precedents, the present doctoral thesis is intended to achieve the following objectives:

- Objective 1: determination of the proteome and immunome changes among *N. caninum* virulent and attenuated isolates.

  The Nc-Liv, Nc-Spain7 and Nc-Spain1H isolates display obvious differences in their virulence. Hence, their proteome (sub-objective 1.1) and immunome (sub-objective 1.2) were compared by 2-DE analyses in order to determine potential virulence factors and antigens involved in the infection mechanisms. Differences in protein abundance among isolates were quantified through DIGE and identified by MALDI-TOF MS techniques. In addition, differentially recognized antigens among the three isolates by sera from experimentally infected mice were also studied.

- Objective 2: description and characterization of the NcROP40 and NcNTPase proteins along the tachyzoite lytic cycle through *in silico* and *in vitro* approaches.

  The rhoptry protein NcROP40 and the dense granules protein NcNTPase showed to be more abundantly expressed in virulent isolates of *N. caninum*. Both proteins were analysed with bioinformatic tools and extensively characterized by a number of *in vitro* approaches. These included the determination of their subcellular localization, the study of their secretion and expression dynamics along the lytic cycle, the assessment of their phosphorylation state during the egress and the analysis of the presence of isoforms or protein species of the NcNTPase protein (sub-objectives 2.1 and 2.2).

- Objective 3: evaluation of the usefulness of NcROP40 and NcNTPase-based vaccines in single and polyvalent formulations combined with the NcROP2Fam-1 and NcGRA7 proteins in a mouse model of neosporosis.

  The ability of NcROP40 and NcNTPase to induce protective immunity against a challenge infection with *N. caninum* was assessed in a pregnant and cerebral mouse model of neosporosis. Proteins were inoculated alone or in combination with the previously tested candidates NcROP2Fam-1 and NcGRA7. Consequently, safety and efficacy for all the formulations were tested through an objective scoring system to evaluate the side effects observed after vaccination and the clinical signs displayed after parasite challenge.

RESULTADOS/RESULTS

OBJETIVO 1/OBJECTIVE 1

Determinación de los cambios en el inmunoma y en el proteoma de aislados de *N. caninum* de alta y baja virulencia.

Determination of the proteome and immunome changes among *N. caninum* virulent and attenuated isolates.

Sub-objective 1.1:

Proteome expression changes among virulent and attenuated *Neospora caninum* isolates

Sub-objective 1.2:

Neospora caninum tachyzoite immunome study reveals differences among three biologically different isolates

Resumen

La neosporosis bovina es la principal causa de aborto de etiología transmisible a nivel mundial en el ganado bovino. La patogenia de la enfermedad se encuentra determinada por factores dependientes del hospedador y del parásito, entre los que destaca la virulencia del aislado. Hasta la fecha, se han descrito variaciones en el perfil genético, el comportamiento *in vitro* y la patogenicidad *in vivo* entre los diferentes aislados de *N. caninum*, aunque los factores responsables de esta diversidad intra-específica del parásito no se han podido identicar.

Con el fin de mejorar el conocimiento sobre los mecanismos del parásito asociados a la virulencia, en el presente objetivo se llevó a cabo un análisis comparativo del proteoma y el inmunoma de tres aislados de alta (Nc-Liv y Nc-Spain7) y baja (Nc-Spain1H) virulencia mediante técnicas de DIGE y Western-blot bidimensional, respectivamente. Las proteínas que mostraron diferencias en su abundancia entre aislados, así como aquellos antígenos diferencialmente reconocidos, fueron identificados mediante técnicas de MALDI-TOF MS.

Los estudios de DIGE mostraron diferencias significativas en la abundancia de ciertas proteínas entre los aislados analizados. Dichas proteínas se asociaron con la motilidad del parásito (NcACT1 y NcMLC1), los componentes de las organelas de secreción (NcMIC1, NcROP9, NcROP40 y NcNTPasa), el metabolismo del parásito (glucosa-6-fosfato deshidrogenasa y 6-fosfogluconato deshidrogenasa), y otras funciones diversas (canal aniónico voltaje-dependiente y aspartil-ARNt sintetasa). No obstante, sólo NcMIC1,

NcROP40, NcNTPasa, glucosa-6-fosfato deshidrogenasa y aspartil-ARNt sintetasa fueron más abundantes en los aislados de alta virulencia, lo que podría determinar la eficacia de sus mecanismos patogénicos en el hospedador.

El análisis del inmunoma se abarcó mediante un diseño 3x3, en el que el extracto proteico de cada uno de los tres aislados se enfrentó a sueros de ratones infectados con los mismos aislados del parásito. En este sentido, las diferencias en el perfil antigénico entre aislados dependieron exclusivamente del suero empleado y no del extracto proteico. Tales diferencias podrían deberse al modo en el que cada aislado es capaz de estimular a la respuesta inmunitaria del hospedador infectado. De este modo, los sueros procedentes de los animales infectados con aislados de alta virulencia mostraron el mayor reconocimiento de antígenos sobre cualquiera de los extractos proteicos empleados, mientras que los sueros de los ratones infectados con el aislado de baja virulencia detectaron un menor número de manchas proteicas. En concreto, la proteína NcGAP45, relacionada con la motilidad del parásito, NcGRA1, presente en los gránulos densos, y la serina-treonina fosfatasa 2C y la superóxido dismutasa, relacionadas ambas con el metabolismo, no fueron reconocidas por dichos sueros.

El presente objetivo abre nuevas vías en la identificación de potenciales dianas vacunales frente a la neosporosis bovina. De tal modo, aquellas proteínas diferencialmente identificadas entre aislados y exclusivamente presentes en los parásitos apicomplejos (NcMIC1, NcGRA1, NcROP40 y NcNTPasa) aparecen como candidatos vacunales preferentes. Del mismo modo, las diferencias encontradas en el patrón antigénico de reconocimiento entre aislados permiten sentar las bases para la identificación de posibles marcadores de virulencia en animales infectados. No obstante, su utilidad debería ser valorada empleando para ello sueros de origen bovino.

Proteome expression changes among virulent and attenuated *Neospora caninum* isolates

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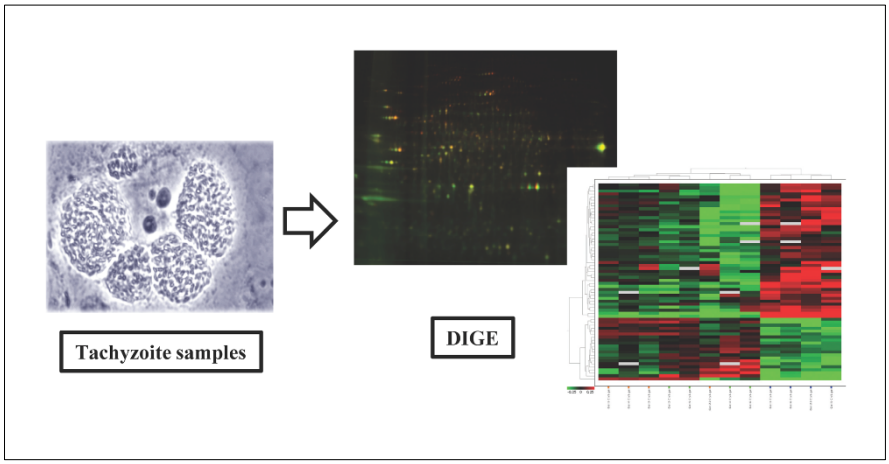

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ABSTRACT

Neospora caninum is a cyst-forming parasite that has been recognised worldwide as a cause of cattle abortion and neuromuscular disease in dogs. Variations in genetic profiles, behaviour *in vitro*, and pathogenicity have been established among *N. caninum* isolates. However, it is unclear which parasite factors are implicated in this intra-specific diversity. Comparative analysis of protein expression patterns may define the determinants of biological diversity in *N. caninum*. Using DIGE and MALDI-TOF MS techniques, we quantified and identified differentially expressed proteins in the tachyzoite stage across three *N. caninum* isolates: the virulent Nc-Liv and Nc-Spain7 isolates, and the attenuated Nc-Spain1H isolate. Comparison between Nc-Spain7 and Nc-Spain1H extracts revealed 39 protein spots that were more abundant in Nc-Spain7 and 21 in Nc-Spain1H. Twenty-four spots were also increased in Nc-Spain7 and 12 in Nc-Liv. Three protein spots were more abundant in the Nc-Liv extracts than in the Nc-Spain1H extracts. MS analysis identified 11 proteins differentially expressed that are potentially involved in gliding motility and the lytic cycle of the parasite, and oxidative stress. These differences could help to explain variations in behaviour between isolates and provide a better knowledge of mechanisms associated with virulence.

1. Introduction

Neospora caninum is a cyst-forming intracellular obligate parasite that is phylogenetically related to *Toxoplasma gondii* and has been recognized worldwide as a cause of neuromuscular disease in dogs and abortion in cattle (Dubey *et al.*, 2007b; Dubey *et al.*, 2006). Advances in studying the *N. caninum* life cycle have proven that canids are the intermediate and definitive hosts, and cattle and other ungulates are the natural intermediate hosts (Dubey *et al.*, 2007a; Dubey & Schares, 2006; Wapenaar *et al.*, 2006). Three infectious stages in its complex life cycle are known: tachyzoite, bradyzoite, and sporozoite. Tachyzoite is the fast-replicating form of the parasite, and this form is responsible for dissemination within the intermediate host during acute infection. Tachyzoites are most often associated with clinical disease and abortion when the parasite is transplacentally transmitted to foetuses during pregnancy after a primary infection (exogenous transplacental infection) or a recrudescence of a chronic infection (endogenous transplacental infection). Consequently, infection and proliferation of parasite in the foetus can produce abortion, birth of a calf with nervous disorders, or birth of a clinically healthy but persistently infected calf that could transmit the parasite to future offspring (Dubey & Schares, 2011; Dubey *et al.*, 2006).

Several authors have reported the influence of *N. caninum* isolate in the outcome of the infection and vertical transmission rates in experimental mouse models (Pereira García-Melo *et al.*, 2010; Regidor-Cerrillo *et al.*, 2010a; Rojo-Montejo *et al.*, 2009b; Collantes-Fernández *et al.*, 2006b; Miller *et al.*, 2002; Quinn *et al.*, 2002b; Atkinson *et al.*, 1999). Furthermore, marked differences in the capacity to produce pathology and abortion have been described

between *N. caninum* isolates using a pregnant bovine model (Rojo-Montejo *et al.*, 2009a; Gibney *et al.*, 2008; Williams *et al.*, 2000). Investigation of the *in vitro* behaviour of a *N. caninum* population has recently demonstrated the potential association of the invasion rates and tachyzoite yields in cell cultures with their pathogenicity in a pregnant mouse model (Regidor-Cerrillo *et al.*, 2011).

Extensive genetic diversity has been also observed in *N. caninum*, but molecular markers employed for genotyping have not allowed discernment of intra-species subgroups associated to pathogenicity (Regidor-Cerrillo *et al.*, 2011; Regidor-Cerrillo *et al.*, 2010a; Beck *et al.*, 2009; Regidor-Cerrillo *et al.*, 2006). This is in contrast to *T. gondii*, which contains genetically polymorphic virulence factors, such as the rhoptry proteins ROP5, ROP18 and ROP16 (Behnke *et al.*, 2011; Reese & Boothroyd, 2011; El Hajj *et al.*, 2007a; Saeij *et al.*, 2006; Taylor *et al.*, 2006). Thus, molecular basis and identification of elements implicated in biological processes of *N. caninum* are unresolved, and finding these elements would explain the biological and pathogenic diversity observed in *N. caninum* isolates.

Proteomic approaches are useful for investigating protein profiles of parasite stages and understanding the biological processes involved in each developmental stage (Marugán-Hernández *et al.*, 2010). Conventional two-dimensional electrophoresis (2-DE) has been previously employed to establish tachyzoite proteome comparisons between *N. caninum* isolates (Lee *et al.*, 2005; Shin *et al.*, 2005b). In these comparative studies, a limited number of proteins were specifically detected for each isolate. However, the functional role and the potential contribution of these proteomic differences to virulence were not discerned.

In the present study, we performed a comparative proteomic approach using Difference Gel Electrophoresis (DIGE) coupled to mass spectrometry (MS) to investigate the differences in abundance of proteins in the tachyzoite stage of three *N. caninum* isolates that displayed different pathogenicity *in vivo* and behaviour *in vitro.* Thus, the virulent Nc-Liv -as reference- and Nc-Spain7 isolates were compared to the low virulent isolate Nc-Spain1H (Regidor-Cerrillo *et al.*, 2011; Regidor-Cerrillo *et al.*, 2010a; Rojo-Montejo *et al.*, 2009a; Rojo-Montejo *et al.*, 2009b; Gibney *et al.*, 2008; Williams *et al.*, 2000).

2. Material and methods

2.1. Parasites

N. caninum tachyzoites from isolates Nc-Liv (Barber *et al.*, 1995), Nc-Spain7 (Regidor-Cerrillo *et al.*, 2008) and Nc-Spain1H (Rojo-Montejo *et al.*, 2009b) were maintained in MARC-145 monolayer following standard procedures (Regidor-Cerrillo *et al.*, 2010a). Tachyzoites used in proteomic analysis were recovered from 3.5-day growth cultures when the majority of them were still intracellular (at least 80% of undisrupted parasite vacuoles in the cell monolayer) and purified using PD-10 (Sephadex G-25) desalting columns (GE Healthcare, Buckinghamshire, UK). Then, tachyzoites were counted in a Neubauer chamber, pelleted by centrifugation at 1,350 x g for 10 min and stored

at -80 ºC until use. Microscope observations of purified tachyzoites were carefully carried out to detect and discard parasite batches with host cell contamination. Four different batches of *N. caninum* pellets from each isolate were obtained according to the required number of replicates for DIGE technology. Tachyzoites were used with a limited number of culture passages (Nc-Liv, passage 24-27; Nc-Spain7, passage 14-17; Nc-Spain1H, passage 27-30). The Nc-Liv isolate was previously passaged in mouse and re-isolated in MARC-145 cell cultures as described (Regidor-Cerrillo *et al.*, 2008). Nc-Liv passage number was determined during cell culture re-isolation.

2.2. Tachyzoite protein extraction for DIGE

Frozen purified tachyzoites (1 x 10^8/batch) were resuspended in 50 µl of lysis buffer containing 6 M urea, 2 M thiourea, 4% (3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris-ClH pH 8.5 and 1 mM phenylmethylsulfonyl fluoride (PMSF) and processed by rapid freeze-thaw in liquid nitrogen. Tachyzoites were further disrupted by two sonications for 15 min each at 4 ºC in a water bath sonicator (Ultrasons-Selecta, Abrera, Spain). Solubilisation was improved by subsequent addition of 300 µl of lysis buffer, and the lysates were maintained in an ice slurry for 1 h with briefly vortexing every 5 min. Insoluble material was removed by centrifugation at 13,000 rpm for 30 min at 4 ºC. All protein extracts used for DIGE analysis were simultaneously obtained.

Protein solutions were precipitated using 2-D Clean-Up Kit (GE Healthcare) following the manufacturer's recommended protocols and resuspended in 50 µl DIGE solution (7 M urea, 2 M thiourea, 30mM Tris-ClH pH 8.5, 4% CHAPS). Final protein concentration was measured by the Bradford method using BSA as the standard.

2.3. Cy dye labeling

Tachyzoite protein extracts were labelled using the fluorescent cyanine dyes (two with Cy3 and two with Cy5 for each isolate extract) according to the manufacturer's instructions (GE Healthcare). Briefly, 50 µg of protein from each sample was labelled with 400 pmol of amine-reactive cyanine dyes freshly dissolved in DMF. The mixture was incubated for 30 min at 4 ºC in the dark, and reactions were quenched by addition of 10 mmol of lysine, which was then followed by incubation for 15 min at 4 ºC in the dark. All DIGE gels included an internal standard, consisting of a pool of equal amounts of protein from each sample included in the experiment that was labelled with Cy2 dye. All protein extracts from all isolate samples were represented in the internal standard (Alban *et al.*, 2003).

2.4. DIGE

Pair wise comparisons were performed according to experimental design developed in this study for DIGE analysis, as shown in Table 1. A total of 150 µg of protein containing 50 µg of the internal standard (Cy2-labelled) and 50 µg of protein extract from Nc-Liv tachyzoites (Cy3-labelled for gels 2 and 3, Cy5-labelled for gels 1 and 4), 50 µg of protein extract from Nc-Spain7 tachyzoites (Cy3-labelled for gels 1 and 6, Cy5-labelled for gels 2 and 5) or 50 µg of protein extract from Nc-Spain1H tachyzoites (Cy3-labelled for gels 4 and 5, Cy5-labelled for

gels 3 and 6) were mixed, and an equivalent volume of loading buffer was added (8 M urea, 4% CHAPS, 130 mM DTT, 2% IPG buffer, pH 3-10).

Isoelectric focusing (IEF) was carried out by using an Ettan IPGphor II unit (GE Healthcare). Samples were loaded into 24-cm non-linear pH 3–11 IPG strips (GE Healthcare) by anodic cup loading and placed on a manifold. Strips were previously hydrated overnight with 0.45 mL hydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 10 mM DTE, 2% IPG buffer, pH 3-10 and blue bromophenol traces), and overlaid with dry strip cover fluid. IEF was carried out for 75 kVh. After IEF, the IPG strips were reduced at room temperature for 12 min in buffer containing 6 M urea, 100mM Tris-ClH pH 6.5, 30% glycerol, 2 % SDS and 0.5 % DTT and alkylated for 5 min in the above buffer, but with 4.5% iodoacetamide in place of DTT. Strips were sealed with 1% agarose to 10% polyacrylamide gels and proteins were separated (2 W/gel for 30 min; 20 W/gel for 4h at 18 ºC) in an Ettan DALTsix Electrophoresis System unit (GE Healthcare).

Table 1: DIGE experimental design. Each gel had three samples, two corresponding to a *N. caninum* isolate sample (labelled with Cy3 and Cy5), and one corresponding to a pooled standard that was common on all gels (labelled with Cy2).

Gel number	Nc-Liv	Nc-Spain7	Nc-Spain1H	Internal standard
Gel 1	Cy5	Cy3		Cy2
Gel 2	Cy3	Cy5		Cy2
Gel 3	Cy3		Cy5	Cy2
Gel 4	Cy5		Cy3	Cy2
Gel 5		Cy5	Cy3	Cy2
Gel 6		Cy3	Cy5	Cy2

2.5. Gel Image analysis and statistics

Image acquisition of fluorochrome-labelled proteins was generated with laser excitation at 488, 532 and 633 nm and emission filters of 520, 580 and 670 nm for Cy2, Cy3 and Cy5 fluorochromes, respectively, using a Typhoon 9400 fluorescence scanner (GE Healthcare). Image cropping and filtering were carried out with Image Quant v. 5.2 software (GE Healthcare) and image analyses for detection of different abundance between spots on the same gel from different tachyzoite extracts were performed with the DIA (Differential In-gel Analysis) module of the DeCyder 6.5 package (GE Healthcare). Relative protein abundance of a spot was defined as the normalized spot volume observed in the Cy3 or Cy5 channel (protein from a particular *N. caninum* isolate) divided by the normalized spot volume of the same spot measured in the Cy2 channel (protein from internal standard pool) on the same gel (Fodor *et al.*, 2005). This value was used in comparisons by the one way-ANOVA and *t*-test statistical analysis. Protein spots displaying a p-value < 0.05 by ANOVA test among the tree isolates were further analyzed in couples using Student's *t*-test. Protein spots with p-value < 0.05 by *t*-test analysis that showed an average 1.4 fold

increase or decrease in their relative abundance in any comparison between two isolates were considered as differentially expressed. Spot matching across the gels was later confirmed by manual inspection. Spots of interest identified through these analyses were verified to have three-dimensional profile characteristics of a protein spot, and features detected from non-protein sources (particles and backgrounds) were filtered out. Only spots that were successfully matched on > 80% (> 15 out of 18) of the gel images were considered.

2.6. Multivariate analysis

The Extended Data Analysis (EDA) module from DeCyder software v6.5 was used to perform a hierarchical cluster analysis including only the proteins that were significantly differentially expressed among *N. caninum* isolates (± 1.4 fold change, ANOVA and *t*-test; $p < 0.05$). The Euclidean method together with an average linkage was used to calculate the distances and linkage, repectively, for the hierarchical clustering.

2.7. Protein staining and in-gel digestion.

Colloidal Coomassie blue staining was used to visualize CyDye-labelled protein features in DIGE gels. Differentially expressed spots were excised from gels and proteins selected were in-gel reduced, alkylated and digested with trypsin (Sechi & Chait, 1998). Briefly, spots were washed twice with water, shrunk for 15 min with 100% acetonitrile (ACN) and dried in a Savant SpeedVac for 30 min. Then, samples were reduced with 10 mM DTE in 25 mM ammonium bicarbonate for 30 min at 56ºC and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Finally, samples were digested overnight with 12.5 ng/µl sequencing-grade trypsin (Roche Molecular Biochemicals, Mannheim, Germany) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37ºC.

2.8. Protein identification by MALDI-TOF MS

After digestion, supernatant was collected and 1 µl was spotted onto a MALDI target plate, and allowed to air-dry at room temperature. Then, 0.4 µl of a 3mg/ml of α-Cyano-4-hydroxycinnamic acid (CHCA; Sigma Aldrich, St. Louis, MO, USA) in 50% ACN was added to the dried peptide digest spots and again allowed to air-dry at room temperature.

MS fingerprinting was performed in a matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)/TOF mass spectrometer (4700 Proteomics Analyzer; PerSeptive Biosystems) operating in reflector mode with an accelerating voltage of 20.000 V. All mass spectra were calibrated externally using a standard peptide mixture (Sigma Aldrich). For protein identification, monoisotopic peptide masses were compared with National Center for Biotechnology Information non-redundant (NCBI nr) and ToxoDB release-v6.0 databases using the MASCOT algorithm v2.1 (Matrix Science) through the Global Protein Server v3.5 from Applied Biosystems. The apicomplexan-specific ToxoDB database contains *N. caninum* annotated protein sequences from genome sequencing. For MS/MS sequencing analyses, suitable precursors were selected and fragmentation was carried out using CID on 1 Kv ion reflector mode and precursor mass Windows +/- 10 Da. Plate model and default calibrations

were optimized for the MS-MS spectra processing. MASCOT search parameters were as follows: carbamidomethyl cystein as fixed modification and oxidized methionine as variable modification; peptide mass tolerance 50-100 ppm; 1 missed trypsin cleavage site; MS/MS fragments tolerance 0.3 Da. Parameters for the combined search (peptide mass fingerprint and MS/MS spectra) were as described above. Identification was accepted when the probability scores were greater than the MASCOT-determined score, with significance set at *p*-value < 0.05 (2-DE). Correlation of gel region with predicted molecular weight and pI were also considered for protein identification.

When insufficient amounts of differentially expressed spots were present in DIGE gels for MS, preparative 2D gels were run with a total of 400 µg protein and visualized with an MS-compatible Coomassie blue staining. Before MS analysis, DeCyder was used to match spots from the preparative gel with those visualized in DIGE gels for accurate spot correspondence.

2.9. Western blot analysis

Western immunoblotting was used to validate differentially expressed proteins identified by MS. Decreasing amounts of each *N. caninum* isolate protein extract (10 µg, 5 µg and 2.5 µg) were diluted in Laemmli sample buffer (final concentrations: 50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue) and separated by 1D-SDS-PAGE in 12.5% acrylamide gels following standard procedures. After electrophoresis, separated proteins were transferred to 0.2 *µ*m Immun-Blot PVDF membranes (BioRad, Hercules, CA, USA). Membranes were blocked with 5% skim milk in TBS-T (50 mM Tris, pH 8.0, 150 mM NaCl and 0.05% Tween-20) for 1 h. Membranes were then incubated in primary antibody solution in TBS-T for 2 h, washed in TBS-T (3 × 10 min) and then probed with either anti rabbit-IgG (1:12000, Sigma Aldrich) and anti-mouse IgG (1:10000, GE Healthcare) horseradish peroxidase-coupled secondary antibodies. After further washing in TBS-T, proteins were revealed using Immobilon Western Chemiluminescent HRP Substrate, a chemiluminescence method (Millipore, Billerica, MA, USA), and protein signal was quantified using a GS-800 Calibrated Densitometer and Quantity One v. 5.1 software (BioRad).

Immunoblottings were probed with successive primary antibodies. The primary antibodies were mouse anti-TgNTPase (1:500) (Asai *et al.*, 1998) and rabbit anti-TgACT1 (1:3000) (Dobrowolski *et al.*, 1997) that were kindly provided by Dr. Sibley. Rabbit anti-NcROP40 was raised in female New Zealand White rabbits (Harlan Interfauna S.A., Barcelona, Spain) immunized with recombinant NcROP40 protein. Membrane was firstly incubated with anti-NTPase and anti-ROP40 antibodies. Then antibody reactions were removed from PVDF membrane by incubations with stripping buffer (1M Glycine, 1% SDS and 0.05% Tween-20, pH 2.2) twice for 20 min at room temperature each time. Then, membrane was washed twice with TBS-T for 15 min. and blocked again with 5% skin milk in TBS-T. Following stripping, membrane was probed with anti-NcROP40 and TgACT1 antibodies as described above.

Recombinant NcROP40 (rNcROP40) protein used for rabbit immunization was expressed in *Escherichia coli* following the previously described standard procedures (Álvarez-García

et al., 2007). Complete NcROP40 ORF was amplified by PCR using the forward primer ROP8-Fw 5'-CGAGCTCATGGTGAAATCCCTGCACAAG-3' and the reverse primer ROP8-Rv 5'-CCTTAATTAATCACCCCACCACTGAACGC-3', and the amplicon was cloned into SacI-PacI digested pET45b+ plasmid (Novagen, EMD Biosciences, Madison, WI). The resulting expression vector was used to transform *E. coli* Rosetta 2(DE3) pLysS (Novagen) and foreign expression of rNcROP40 protein as a (His)6-tagged fusion protein. The rNcROP40 was then purified by Ni^{2+}-chelate chromatography using a HisTrap FF column (GE Healthcare, Piscataway, NJ), following manufacturer recommends. Protein recognition of rabbit anti-NcROP40 and anti-TgACT1 was checked against *N. caninum* and *T. gondii* extracts (Supplementary Figure 1).

2.10. Ethics statement in animal experimentation

Antibody raising in rabbits was performed under animal-handling procedures that complied with Spanish and EU legislation (Law 32/2007, R.D. 1201/2005, and Council Directive 2010/63/EU). The Animal Research Committee of the Complutense University of Madrid approved all experiments and specific details of our procedures according Spanish and EU legislation, and following proceedings described in Regulation of Internal Regime for Animal Research Committee (published at Boletín Oficial de la Universidad Complutense de Madrid – BOUC-, nº 2, at 9 February 2006).

3. Results and discussion

3.1. Differentially abundant proteins detected across N. caninum *isolates*

Because phenotypic traits are mainly due to the expression, abundance and activation status of proteins, investigating tachyzoite-proteome differences among *N. caninum* isolates may identify proteins and processes implicated in the intra-specific diversity displayed by *N. caninum,* including proteins with roles pathogenicity. In this study, DIGE and MS technology was applied to investigate tachyzoite proteome changes between the Nc-Liv, Nc-Spain1H and Nc-Spain7 isolates that had demonstrated marked differences in *in vivo* and *in vitro* behaviour (Regidor-Cerrillo *et al.*, 2011; Regidor-Cerrillo *et al.*, 2010a; Rojo-Montejo *et al.*, 2009b; Gibney *et al.*, 2008; Williams *et al.*, 2000). DIGE analysis provides advantages of reproducibility and sensitivity, and can be used for relative quantification with statistical analysis. This relative quantification allows us to identify proteins that are differentially expressed between biological samples (Fodor *et al.*, 2005; Marouga *et al.*, 2005). In particular, DIGE technology has been recently applied to *N. caninum* to discern mechanisms involved in the tachyzoite to bradyzoite stage conversion (Marugán-Hernández *et al.*, 2010).

In this study, DIGE image analysis detected more than 2,500 protein spots for each gel (Figure 1). In previous comparative studies, a minor average of 544 (516-573) and 530 (520-540) spots was detected from 2-DE gel stained with silver nitrate using pH 4-7 IPG strips (13 cm) (Lee *et al.*, 2005; Shin *et al.*, 2005b). Analysis of the Cy2, Cy3 and Cy5 gel images and subsequent proteome

comparisons did not detect specific spots for each isolate (isolate-specific spots), confirming a whole protein correspondence across Nc-Liv, Nc-Spain7 and Nc-Spain1H proteomes. Nevertheless, DIA analysis detected 66 protein spots with a significant increase or decrease (± 1.4 fold change) in relative abundance (p<0.05 by ANOVA and *t*-test), demonstrating evidence of marked variations in the tachyzoite protein expression profiles among these three *N. caninum* isolates. Comparison between Nc-Spain7 and Nc-Spain1H proteomes revealed changes in the abundance of 60 protein spots, 39 were more abundant in the Nc-Spain7 proteome and 21 were more abundant in the Nc-Spain1H isolate proteome (Figure 1A). Three protein spots were significantly increased in the Nc-Liv isolate when compared with the Nc-Spain1H proteome (Figure 1B). Finally, up to 36 differentially expressed protein spots were also detected between Nc-Spain7 and Nc-Liv proteomes, 24 were more abundant in the Nc-Spain7 isolate and 12 in the Nc-Liv isolate (Figure 1C). Most of protein expression variations were minor with only a ± 2.5 fold change. However, spots with higher changes (from ± 2.5 to ± 6.7 fold) were also found in the Nc-Spain7 extract comparisons. Five spots were increased with a ± > 2.5 fold change when Nc-Spain7 was compared with both Nc-Spain1H and Nc-Liv extracts, and 2 further spots were found when comparing against the Nc-Spain1H extract (Supplementary Table 1). Thus, Nc-Spain7 isolate showed the highest number of differentially expressed spots and higher expression differences.

The EDA module in DeCyder was used for the multivariate analysis of selected spot dataset. Hierarchical pattern analysis of expression values clearly distinguished Nc-Spain7 pattern from the Nc-Liv and Nc-Spain1H patterns, clustering the different experimental groups with two major branches (Figure 2). Nc-Spain7 samples were clearly segregated in one cluster, and Nc-Liv and Nc-Spain1H samples formed a single major cluster due to minor differences observed in their expression patterns. Nc-Liv and Nc-Spain1H replicates also showed a trend to be grouped together, and only one Nc-Liv sample was intercalated into Nc-Spain1H cluster (Figure 2). Additionally, Nc-Liv and Nc-Spain1H replicates were grouped together when the Pearson method was applied for hierarchical clustering in the multivariate analysis (data not shown).

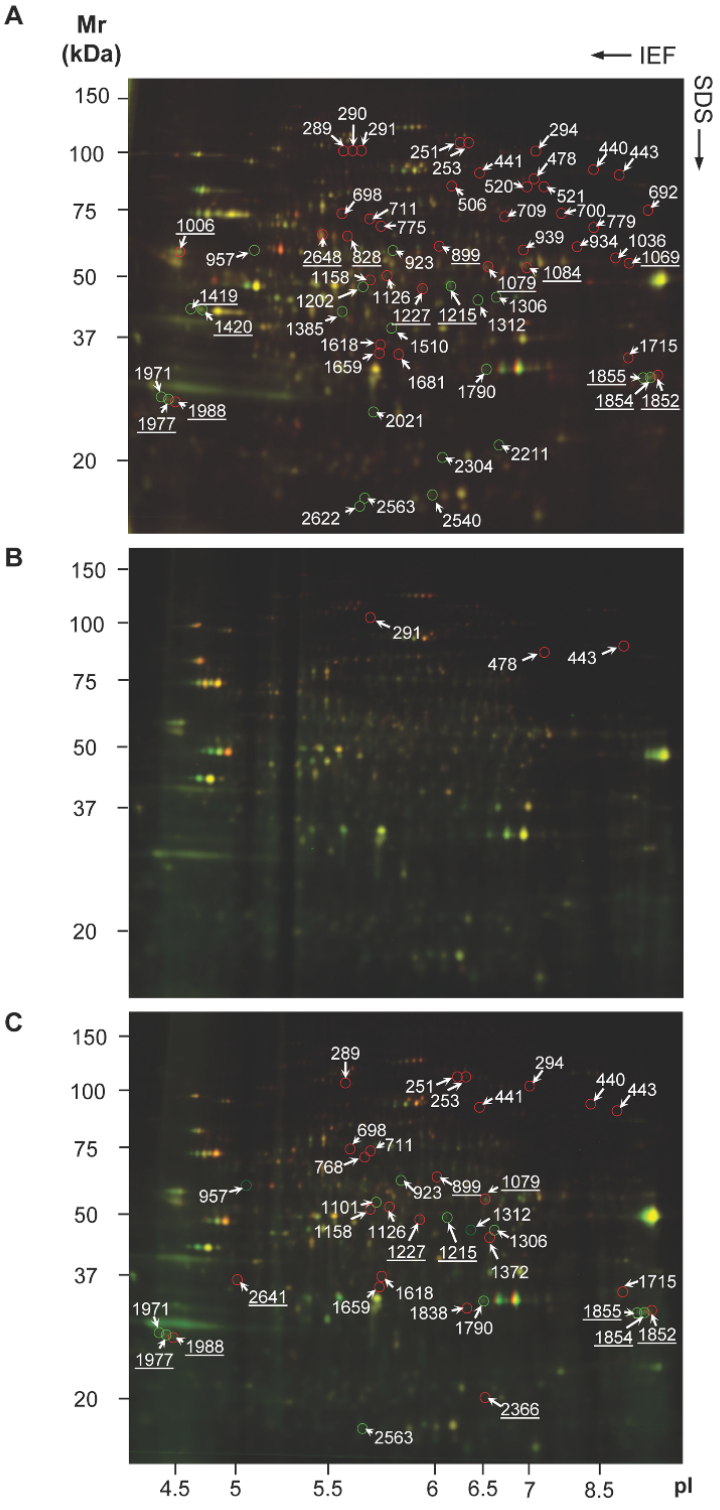

Figure 1: Comparisons across Nc-Spain7, Nc-Liv and Nc-Spain1H tachyzoite protein extracts using DIGE. Representative gels for Nc-Spain7 (Cy5 labelled) *versus* Nc-Spain1H (Cy3 labelled) (Figure 1A), Nc-Liv (Cy5 labelled) *versus* Nc-Spain1H (Cy3 labelled) (Figure 1B) and Nc-Spain7 (Cy5 labelled) *versus* Nc-Liv (Cy3 labelled) (Figure 1C) are showed. Open circles indicate identified protein spots significantly increased by more than 1.4-fold or decreased by more than 1.4-fold. The number near the circle is the spot number, as indicated in Table 2, Table 3 and Supplementary Table 1. Underlined spot numbers are protein spots identified by MS.

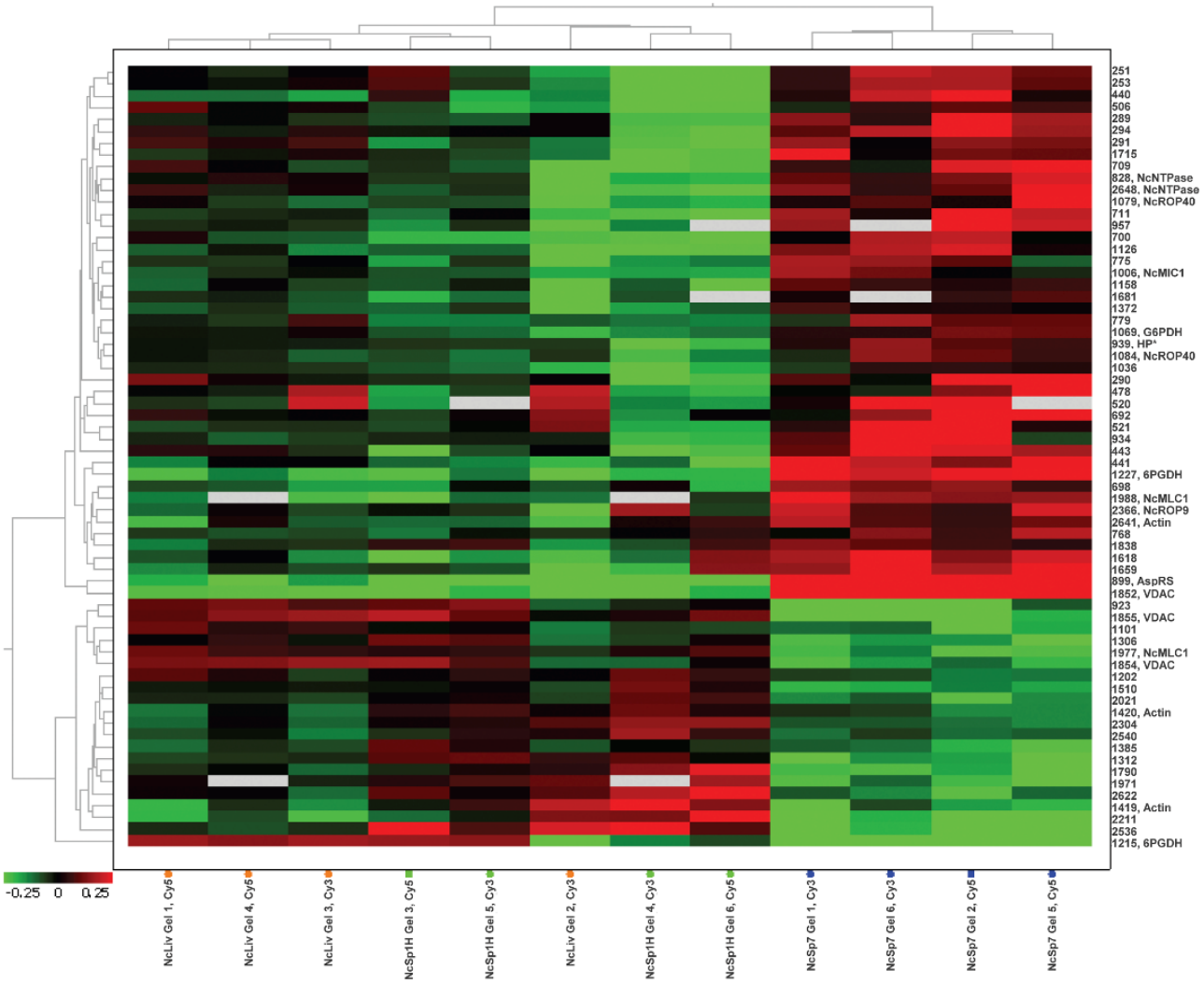

Figure 2: Hierarchical clustering of the 12 samples based on the global expression pattern of selected spots (Supplementary Table 1). The dendrogram on top shows the degree of similarity in protein expression patterns between the *N. caninum* isolates, which are represented in columns. The dendrogram of individual proteins is shown on the left, with relative expression values displayed as heat map using a standardized log abundance scale ranging from -0.25 (down-regulated; green) to +0.25 (up-regulated; red). The isolate sample, gel number and dye labelling for each sample are listed below, and the spot number and protein identification are listed along the right-hand side. Two main clusters of the protein patterns were displayed grouping separately the Nc-Spain7 from Nc-Liv and Nc-Spain1H samples.

3.2. Protein identification

Most differentially expressed spots were lowly abundant proteins because only 33 of 66 differentially expressed spots by DIGE could be visualized by Coomasie staining and excised from gels for MS analysis. Nineteen protein spots were unambiguously identified out of 33 picked spots (Table 2 and 3). When spectra data were compared against NCBI nr database, eight spots were identified as *Neospora* or *Toxoplasma* proteins (data not shown). Identifications notably increased when mass fingerprints were compared against ToxoDB 6.0 database, and 18 protein spots were identified. MS/MS combined with MS identified 1 new protein spot. The identification of these 19 spots corresponded

to 11 proteins because different spots were recognized as the same protein, indicating that these protein species may involve multiple isoforms or post-translationally modified forms (Table 2 and 3). Therefore, when different spots identified as the same protein showed significant increases or decreases in abundance according to the assessed spot, a reliable assignment of up- or down-regulation could not be established for this protein. Even so, shifts in relative abundance of specific spots for the same protein are likely to reflect functional significance.

Actin was identified in three spots, two exhibited the same Mr, whereas the third showed a lower Mr in the gel. Actin abundance varied in these three protein spots according to the isolate (Table 2). The predicted amino acid sequence of *N. caninum* actin (NcACT1) has a 100% identity with its homologue in *T. gondii* (TgACT1) according to sequence alignment carried out in this study (data not shown), suggesting that functionality and regulation could be similar for actin proteins in both species. In addition, two different spots were identified as the myosin light chain protein of *N. caninum* (NcMLC1) according to the corresponding sequence deposited in ToxoDB database. In *T. gondii,* the TgMLC1 orthologue shows 82% identity with NcMLC1, and interactions between myosin MyoA with TgGAP45-TgGAP50 proteins anchor this motor complex to the inner membrane complex (IMC) (Daher & Soldati-Favre, 2009; Gilk *et al.*, 2009; Carruthers & Boothroyd, 2007). Gliding motility, essential for lytic cycle, is mediated by this actin/myosin-based motor complex anchoring to the IMC in *T. gondii.* Both mechanisms, control over the time and localisation of actin filament polymerization and TgMLC1 regulation, have been described as modulation keys for parasite motility, invasion and other processes, such as parasite replication (Mehta & Sibley, 2011; Heaslip *et al.*, 2010; Agop-Nersesian *et al.*, 2009; Daher & Soldati-Favre, 2009; Gilk *et al.*, 2009; Carruthers & Boothroyd, 2007; Sahoo *et al.*, 2006). Consequently, NcACT1 and NcMLC1 proteins could play similar crucial roles in these essential *N. caninum* processes.

NcMIC1 is a secretory protein localised in micronemes that is released immediately after adhesion, and it acts to stabilise the interaction between tachyzoite and host cell during invasion (Keller *et al.*, 2002). In this study, NcMIC1 protein showed also different abundances between isolates (Table 2). In *T. gondii,* TgMIC1 assembles with other microneme secreted proteins, TgMIC4 and TgMIC6, to form one of the three major complexes involved in invasion. In fact, Tg*MIC1* deletion causes a drastic decrease in tachyzoite invasion. Moreover, deletion of both Tg*MIC1* and Tg*MIC3*, another microneme component, leads to a decrease in virulence in *T. gondii* (Cerede *et al.*, 2005). Decreased abundance of NcMIC1 for Nc-Spain1H isolate may be associated with their reduced invasiveness displayed *in vitro* and their pathogenicity *in vivo* (Regidor-Cerrillo *et al.*, 2011; Rojo-Montejo *et al.*, 2009b).

During cell invasion, rhoptries organelles also secrete its contents after microneme draining. In *T. gondii* rhoptry proteins includes RON proteins, that embrace some elements implicated in the creation of moving junction for parasite internalisation, and ROP proteins, dominated by a large family of proteins with conserved serine/threonine kinases domains that have been associated with the development and

maintenance of nascent parasitophorous vacuole, and regulation of host cell processes (Qiu *et al.*, 2009; Boothroyd & Dubremetz, 2008). In *T. gondii*, three ROP proteins; ROP5, ROP16 and ROP18 have been recognized as virulence factors (Behnke *et al.*, 2011; Reese & Boothroyd, 2011; El Hajj *et al.*, 2007a; Saeij *et al.*, 2006; Taylor *et al.*, 2006). Furthermore, ROP5 proteins are indispensable for *Toxoplasma* to cause disease in mice (Behnke *et al.*, 2011; Reese & Boothroyd, 2011). In this study, two orthologues for ROP proteins were differentially expressed among *N. caninum* isolates. Two proteins species identified as the *N. caninum* orthologue of the TgROP40 protein were more abundant in the Nc-Spain7 isolate. Furthermore, one protein species (spot 2648) was also slightly increased with statistical significance in the Nc-Liv isolate (+ 1.22 fold change; $p<0.05$) compared with the Nc-Spain1H (Table 2). NcROP40 that was previously assigned as the NcROP8 orthologue in ToxoDB v5.2 protein database (psu|NC_LIV_000700), has been also recently identified by LC/MS-MS analysis of subcellular fractions enriched in rhoptry proteins (Marugán-Hernández *et al.*, 2011a). Additionally, NcROP9 was more abundant in Nc-Spain7 than in Nc-Liv (Table 2). However, the functionality for these ROP proteins is unknown in *N. caninum* and limited in *T. gondii* (Qiu *et al.*, 2009; Reichmann *et al.*, 2002). It is possible that they could also play different roles in the two parasites, as suggested in a previous study (Phelps *et al.*, 2008).

Dense granule proteins are secreted during and after invasion into the parasitophorous vacuole, and they either remain in the lumen or are associated with the tubulovesicular network and membrane for the nutrient acquisition by the parasite (Nam, 2009; Mercier *et al.*, 2005). NcNTPase, which is a nucleoside triphosphate hydrolase (EC 3.6.1.15) activated by dithiols, is also secreted from dense granules (Asai *et al.*, 1998). In this study, NcNTPase was identified in two spots that were significantly less abundant in the Nc-Spain1H than in the Nc-Spain7 isolate (Table 2). Additionally, protein expression of NcNTPase was also slightly increased for one spot in the Nc-Liv protein extracts when compared to Nc-Spain1H (+ 1.27 fold change; $p< 0.05$). Two NTPases have been identified in *T. gondii* named as TgNTPase-I and TgNTPase-II, whose functionality was implicated in purine salvage (Mercier *et al.*, 2005; Asai *et al.*, 1995; Bermudes *et al.*, 1994). TgNTPase-I had been associated with virulence because this protein is restricted to virulent Type I isolates of *T. gondii* (Asai *et al.*, 1995). The NcNTPases are also encoded by at least two genes in *N. caninum,* but the two proteins display a higher similarity to *T. gondii* NTPase-I with hydrolytic activity for triphosphate nucleosides but non-measurable hydrolysis activity for diphosphate nucleosides, which discards their implication in purine salvage (Asai *et al.*, 1998). The NcNTPase protein sequences expressed in the tachyzoite stage only differ in four amino acids (Asai *et al.*, 1998). Due to this limited number of amino acid polymorphisms for NcNTPases, the analysis of sequences of predicted peptides by MS for each NTPase spot (828 and 2648) did not allow us to discern with accuracy which NcNTPase was significantly increased in Nc-Spain7 and Nc-Liv isolates. Nevertheless, NcNTPase activity may be necessary for the survival and proliferation of the parasite during intracellular development, as in *T. gondii* (Asai *et al.*, 2002; Nakaar *et al.*, 1999), and it

could be involved in egress of the parasite (Esposito *et al.*, 2007; Silverman *et al.*, 1998).

Alterations in abundance of oxidative stress enzymes were also detected across the three isolates in this study. Glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) expression was more abundant in the Nc-Spain7 protein extract than in the Nc-Spain1H protein extract. The 6-phosphogluconate dehydrogenase enzyme (6PGD; EC 1.1.1.44) was identified from two different spots that significantly varied between the Nc-Spain7 protein extract and both the Nc-Spain1H and Nc-Liv protein extracts (Table 2). Both proteins operate in the oxidative branch of the pentose phosphate pathway (PPP) to generate reducing power in the form of NADPH (Figure 3). Because PPP was not previously described in *N. caninum*, we used the amino-acid sequences of predicted enzymes involved in PPP from *T. gondii* (KEGG Pathway tgo00030 site accessed via ToxoDB) for a BLASTP search of the ToxoDB v6.0 *N. caninum* database. Key enzymes participating in PPP were thus predicted in *N. caninum*, and the expression of G6PD and 6GPD was confirmed in the tachyzoite-stage (Supplementary Table 2). Similar to *T. gondii*, most of PPP enzymes were encoded only once in the *N. caninum* genome. However, the G6PD and 6GPD predicted enzymes were encoded by two genes that were differed in whether they contained an amine-terminal signal sequence. Therefore, these proteins likely have different cellular locations. The PPP is required for nucleotide and nucleic acid synthesis and for intracellular proliferation of parasite. Moreover, G6PD and 6GPD, through NADPH production, maintain the cellular redox state. Additionally, they may be crucial in protecting the parasite from oxidative stress caused by nitroside oxide (NO) produced in Th1-type immune response of the *Neospora*-infected host (Pinheiro *et al.*, 2010).

Modulation of a porin family member with predicted voltage-dependent anion channel activity (VDAC) was also detected. VDAC was presented in two different spots that were significantly increased or decreased, respectively, in the Nc-Spain7 extract compared with the other isolates (Table 2). VDAC homologues are broadly expressed in all eukaryotes, and they have been recognised the major governor of mitochondrial function. They play a role in the transport of metabolites and ions across the mitochondrial outer membrane, including the ATP/ADP exchange between mitochondria and the cytosol (Peixoto *et al.*, 2010; Rostovtseva & Bezrukov, 2008). Similar physiological role may be attributed to the VDAC-porin in *N. caninum* and *T. gondii*.

Two additional proteins that were over-expressed in the Nc-Spain7 isolate were identified as hypothetical proteins (HPs) because there was not *a priori* functional information for these proteins in the *N. caninum* genome database. One of these proteins showed conservation with a *T. gondii* orthologue that was functionally assigned as an aspartyl-tRNA synthetase (AspRS) protein. Subsequent sequence alignment of *T. gondii* and *N. caninum* AspRSs revealed that the *N. caninum* AspRS is a shorter protein that aligns with the carboxyl-terminal half of *T. gondii* protein with 89% identity (Supplementary Figure 2). A signal peptide was not predicted for NcAspRS protein. Thus, *N. caninum* AspRS may represent a truncated AspRS or incompletely predicted protein. Aminoacyl-tRNA synthetases are ubiquitous enzymes that

join specific amino acids to their cognate tRNAs and, therefore, they are essential for protein synthesis. However, recent investigations have shown that aminoacyl-tRNA synthetases may be involved in alternative functions, such as translation as tRNA processing, RNA splicing, RNA trafficking, apoptosis, and transcriptional and translational regulation (Bour *et al.*, 2009; Park *et al.*, 2005).

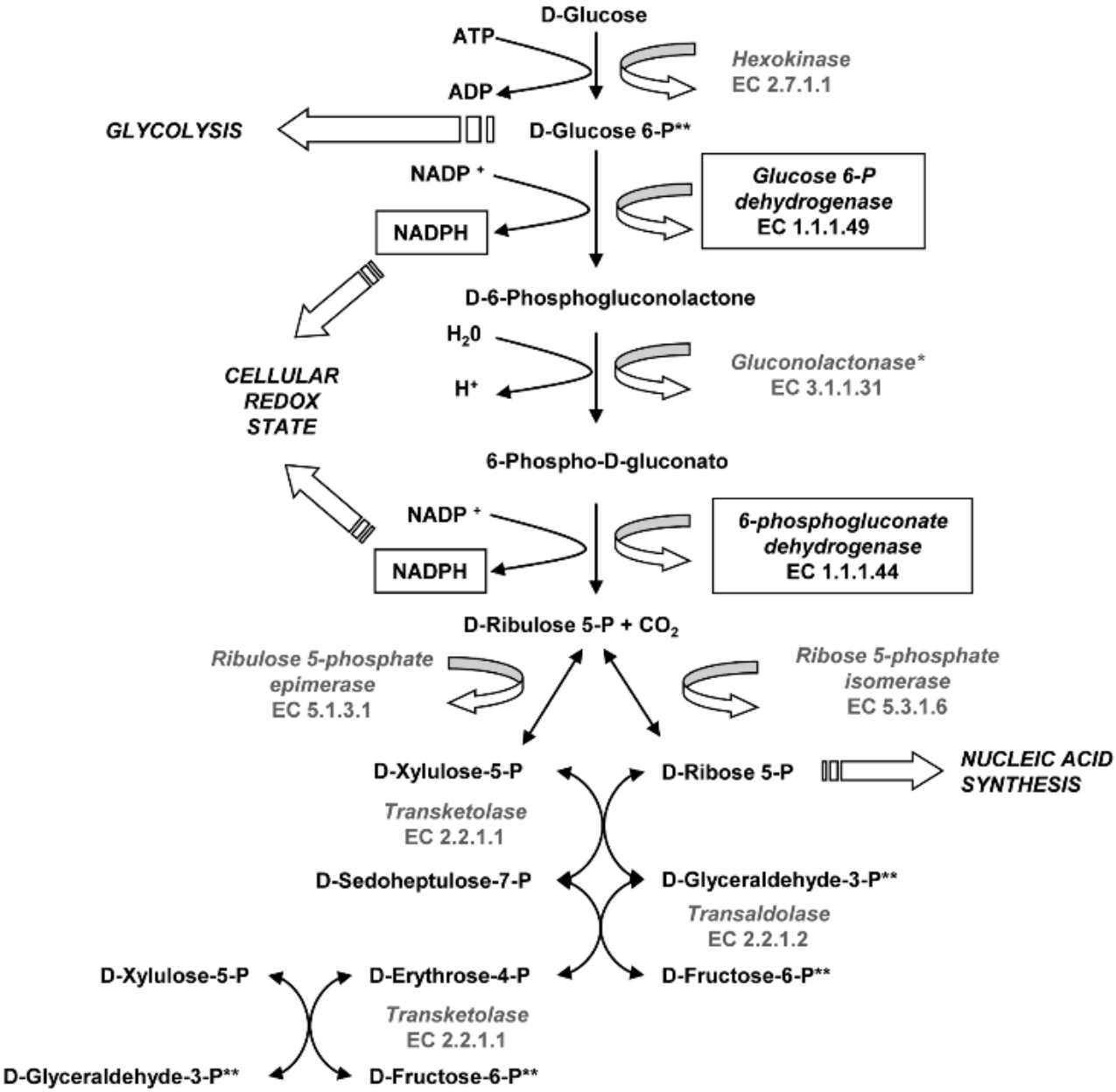

Figure 3: Schematic representation of the pentose phosphate pathway (PPP) with component enzymes predicted to be present in *Neospora caninum*. All orthologous genes that encode PPP enzymes, with the exception of the gene coding for glucolactonase (*), were identified by analysis of the *N. caninum* genome. Genes with MS-confirmed tachyzoite protein expression and differentially abundant across Nc-Liv, Nc-Spain1H and Nc-Spain7 isolates are boxed. ** indicates typical metabolic compounds from glycolysis/gluconeogenesis pathway.

Table 2: Summary of differentially abundant proteins among *Neospora caninum* isolates that were identified by MS.

Identified protein	Spot no.	ANOVA *p*-value	Nc-Spain7 *vs.* Nc-Spain1H comparison		Nc-Liv *vs.* Nc-Spain1H comparison		Nc-Spain7 *vs.* Nc-Liv comparison	
			Average ratio	t-test *p*-value	Average ratio	t-test *p*-value	Average ratio	t-test *p*-value
NTPase	828	0.029	1.76	0.0035	1.17	0.57	1.5	0.074
	2648	0.045	1.98	0.0059	1.27	0.045	1.57	0.099
Aspartyl-tRNA synthetase	899	2.70E-05	6.8	2.80E-05	1.41	0.28	4.74	0.00038
Hypothetical protein	939	0.0034	1.67	0.0076	1.27	0.079	1.31	0.0042
Microneme-associated protein (NcMIC1)	1006	0.015	1.57	0.013	1.11	0.34	1.41	0.045
Glucose-6-phosphate dehydrogenase	1069	0.0068	1.55	0.00025	1.15	0.34	1.35	0.047
ROP40 kinase	1079	0.017	1.7	0.011	1.07	0.73	1.58	0.041
	1084	0.0039	1.62	0.007	1.22	0.046	1.33	0.038
6-P gluconate dehydrogenase	1215	0.00028	-4.67	0.00056	1.1	0.8	-5.16	0.0012
	1227	6.60E-07	2.93	2.10E-05	-1.05	0.55	3.08	2.10E-05
Actin	1419	0.042	-1.96	0.0083	-1.4	0.19	-1.39	0.25
	1420	0.006	-1.45	0.0021	-1.31	0.022	-1.1	0.39
	2641	0.015	1.38	0.037	-1.23	0.23	1.7	0.011
Porin; VDAC	1852	7.00E-07	4.32	1.10E-05	-1.08	0.61	4.65	1.30E-05
	1854	0.014	-1.67	0.015	1.1	0.65	-1.83	0.0098
	1855	7.00E-05	-2.59	0.00043	-1.03	0.8	-2.52	0.00068
Myosin light chain	1977	8.40E-05	-1.8	8.50E-05	-1.01	0.82	-1.78	0.0012
	1988	0.00084	2.07	0.0037	-1.05	0.83	2.18	0.00062
NcROP9	2366	0.023	1.35	0.098	-1.31	0.19	1.76	0.013

Table 3: Summary of differentially abundant proteins identified by MALDI TOF MS and MS/MS analysis.

Identified protein	Spot no.	Accession no. NCBI GenBank	Accession no. ToxoDB	Theo. MW (kDa)	Theo. pI	Protein score	Sequence coverage	No matched peptides[a] or amino acid sequence of peptides[b]	Functional category
NTPase	828	BAI44520	NCLIV_068400	69.61	5.56	141	35%	20/65	Nucleoside-triphosphatase
		BAI31454	NCLIV_068460	110.64	5.29	100	21%	19/65	
	2648	BAI44520	NCLIV_068400	69.61	5.56	144	34%	21/65	
		BAI31454	NCLIV_068460	110.64	5.29	112	21%	21/65	
Aspartyl-tRNA synthetase	899	XP 002367538[c,d]	NCLIV_022400	62.83	5.95	205	48%	22/65	tRNA aminoacylation
						109	23%	R.VFEIGPVFR.A (20) R.LGTAPHGGAGIGLER.V (8)	
Hypothetical protein	939	-	NCLIV_064950[d]	55.02	7.77	92	30%	13/41	-
						95	30%	K.FVGPAAEELER.Q (16)	
Microneme-associated protein (NcMIC1)	1006	AAL37729	NCLIV_043270	50.15	4.79	163	48%	19/65	Tachyzoite invasion
Glucose-6-phosphate dehydrogenase	1069	-	NCLIV_000940[d]	54.81	8.66	106	37%	15/56	Carbohydrate metabolism /redox status
						125	39%	R.GEFFDAYGIIR.D (11)	
ROP40 kinase	1079	-	NCLIV_012920[d]	43.20	6.29	258[d]	77%	26/65	Protein kinase
						294	77%	R.AIQPPFFR.K (17) R.LQFDVGSAQR.V (28)	
	1084	-	NCLIV_012920[d]	43.20	6.29	107[d]	38%	18/65	
						232	**63%**	**R.DGILYFGGFSSK.V (44) R.FTAEDNAADLGR.T (2)**	

Table 3 (continuation): Summary of differentially abundant proteins identified by MALDI TOF MS and MS/MS analysis.

Identified protein	Spot no.	Accession no. NCBI GenBank	Accession no. ToxoDB	Theo. MW (kDa)	Theo. pI	Protein score	Sequence coverage	No matched peptides[a] or amino acid sequence of peptides[b]	Functional category
6-P gluconate dehydrogenase	1215	-	NCLIV_060600	55.29	6.28	107	30%	13/49	Carbohydrate metabolism /redox status
	1227	-	NCLIV_060600	55.29	6.28	194	45%	24/61	
Actin	1419	XP 002369663[c]	NCLIV_003440	42.16	5.05	218	68%	23/65	Tachyzoite motility and invasion
	1420	XP 002369663[c]	NCLIV_003440	42.16	5.05	339	78%	34/65	
	2641	XP 002369663[c]	NCLIV_003440[d]	42.16	5.05	122[d]	50%	19/65	
						120	50%	K.IWHHTFYNELR.V (3)	
Porin; VDAC	1852		NCLIV_024630	31.57	8.87	90	40%	11/65	Metabolite transport
	1854		NCLIV_024630	31.57	8.87	72	41%	9/65	
	1855	XP 002365430[c,d]	NCLIV_024630[d]	31.57	8.87	189	43%	K.SAELAAEVDCNLLDGR.T (105)	
Myosin light chain	1977		NCLIV_029420	24.94	4.55	68	36%	12/65	Tachyzoite motility and invasion
	1988		NCLIV_029420	24.94	4.55	68	36%	11/65	
NcROP9	**2366**		**NCLIV_018420**	**37.27**	**8.71**	**184**	**47%**	**19/65**	Invasion[e]

[a] Number of peptide masses values matched/searched.

[b] Amino acid sequence identified by MS/MS; ion score is indicated in parentheses.

[c] Protein identification data by comparison with *T. gondii* ME49 sequence in the National Center for Biotechnology Information non-redundant (NCBI nr) database.

[d] Protein identification was also performed by MALDI TOF MS/MS analysis.

[e] Protein function assigned according to TgROP9 orthologue (Reichmann *et al.*, 2002).

3.3. Validation of identified proteins by Western blot analysis

Western immunoblot analyses were carried out to confirm differential protein levels for several tachyzoite proteins (NcNTPase, NcROP40 and actin) across Nc-Spain1H, Nc-Spain7 and Nc-Liv isolates (Figure 4). These proteins may play important roles in tachyzoite motility, invasion, intracellular proliferation and virulence in *N. caninum*. NTPase and actin were evaluated using anti-TgNTPase (Asai *et al.*, 1998) and anti-TgACT1 (Dobrowolski *et al.*, 1997), antibodies (Supplementary Figure 1), respectively. Rabbit serum that was raised against purified rNcROP40 protein was used for NcROP40 validation. This polyclonal antibody reacts mainly to a specific band in the *N. caninum* extract which migrates at 53 kDa, which is analogous to the Mr of NcROP40 spots visualized in DIGE gels. While the antibody gave robust detection of ROP40 protein in *N. caninum* extract, cross-reactivity against *T. gondii* protein extract was not observed (Supplementary Figure 1).

The results of the immunoblotting indicate that NcNTPase was more abundant in Nc-Spain7 tachyzoites compared to Nc-Spain1H tachyzoites. Additionally, expression levels of NcNTPase were augmented in the Nc-Liv isolate, as expected from 2D-DIGE results (spot 2648), although 2D-DIGE only demonstrated a < +1.4 fold change (Figure 4A). NcROP40 protein levels were also increased in Nc-Spain7 and Nc-Liv isolates compared with the Nc-Spain1H isolate. Similarly, Nc-Liv isolate showed a significant increase in NcROP40, but with a < +1.4 fold change (spot 1084) in the 2D-DIGE results (Figure 4B). The actin blotting results did not completely agree with the 2D-DIGE data. In contrast to 2D-DIGE, 44 kDa actin species (spots 1419 and 1420) levels in Nc-Spain7 and Nc-Liv isolates were higher than Nc-Spain1H isolate. However, according to 2D-DIGE analysis, expression levels of the 36.5 kDa actin species (spot 2641) were augmented in Nc-Spain7 isolate compared with Nc-Liv isolate. Because 44 kDa actin spots are integrated in a protein-chain of multiple actin species identified in this study (data not shown), variations in actin spots that were not previously selected by DIA analysis (non-statistically, < ± 1.4 fold change) may mask the immunoblotting results for the 44 kDa actin species if the different protein species are not differentiated.

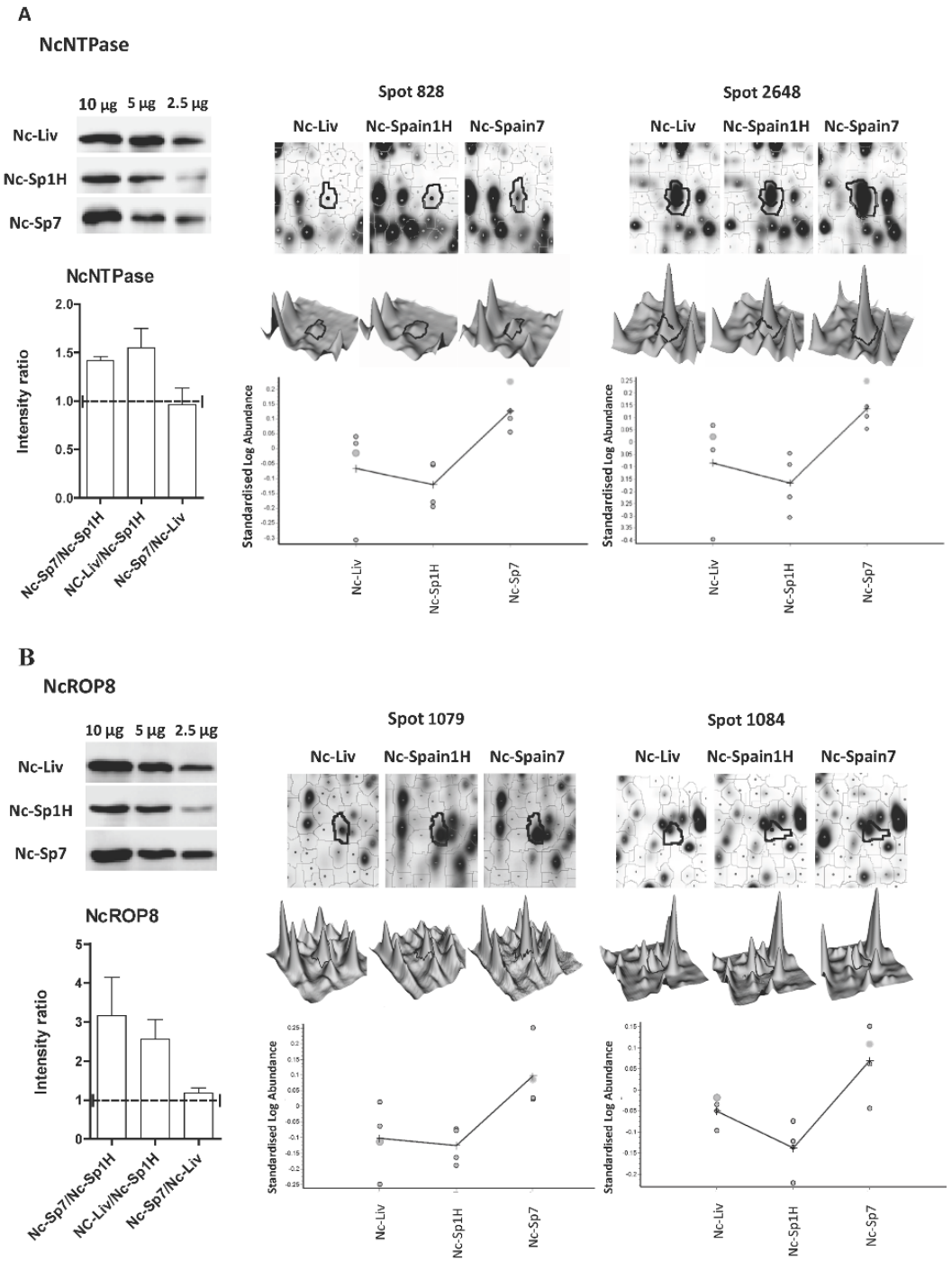

Figure 4: Representative 2D-DIGE image, quantification and western blot analysis for selected differentially expressed proteins across Nc-Spain1H, Nc-Spain7 and Nc-Liv isolates. Magnified gel images, 3D images, statistical analysis (by BVA module), western blot images and density comparisons of identified proteins (A) NcNTPase and (B) NcROP40. Western blotting for selected proteins was performed with decreasing amounts of each protein extract (10 µg, 5 µg and 2.5 µg). Expression levels of identified proteins were assessed by density values (sum of pixel intensities by pixel area). Protein expression differences were determined by intensity ratios obtained from the different protein samples for each isolate. Intensity ratios of NcROP40 (B) were simultaneously assessed with NcACT1, and were similar to those prior determined simultaneously with NcNTPase (data not shown). Error bars represent S.D.

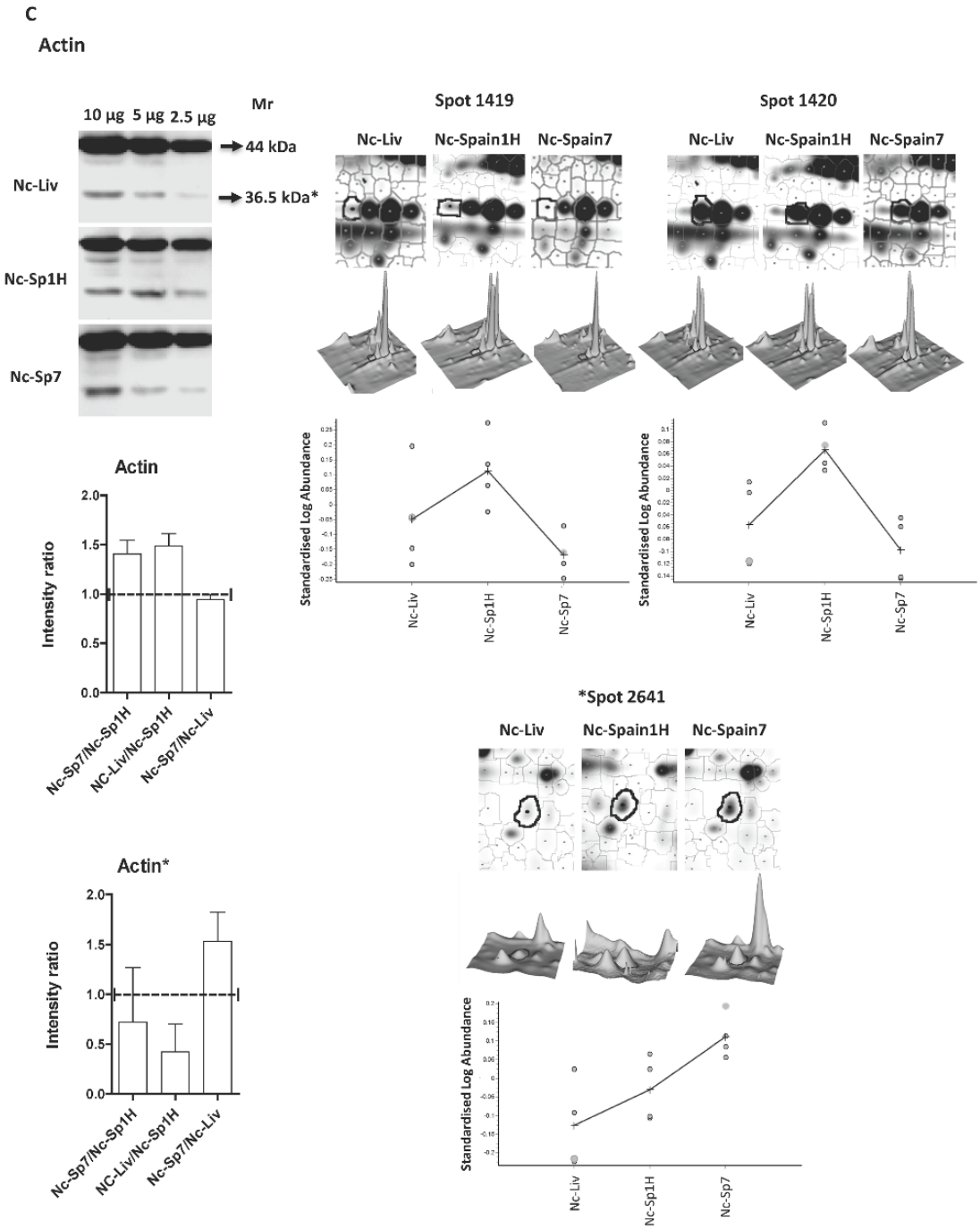

Figure 4 (continuation): Representative 2D-DIGE image, quantification and western blot analysis for selected differentially expressed proteins across Nc-Spain1H, Nc-Spain7 and Nc-Liv isolates. Magnified gel images, 3D images, statistical analysis (by BVA module), western blot images and density comparisons of identified proteins (C) 44 kDa and 36.5 kDa actin species. Western blotting for selected proteins was performed with decreasing amounts of each protein extract (10 µg, 5 µg and 2.5 µg). Expression levels of identified proteins were assessed by density values (sum of pixel intensities by pixel area). Protein expression differences were determined by intensity ratios obtained from the different protein samples for each isolate. Error bars represent S.D.

3.4. Funtional role of differentially expressed proteins in N. caninum *biology*

All protein identifications in this study correlate well with those expected for the invasive and rapidly dividing tachyzoite-phase of *N. caninum*, because elements involved in motility, invasion and intracellular proliferation as apical-organelle proteins, in addition to protein and nucleotide synthesis, mitochondrial trans-membrane transport activity and the oxidative stress response, were identified in this study. Because *in vivo* and *in vitro* behaviour involves the participation of a broad variety of proteins, they may be playing key multifactorial roles in the phenotypic differences between the three *N. caninum* isolates. Thus, a decreased expression of several dense granule proteins –TgGRA1-5, TgGRA7 and TgGRA8–, microneme protein TgMIC5, TgNTPase I, catalase and TgACT1, was recognized as cause of virulence attenuation of a Type I *T. gondii* isolate that was adapted by successive passaging through cell culture (Nischik *et al.*, 2001). Notably, reduced expression of NcNTPase, microneme protein NcMIC1, and NcROP40 proteins may be involved in the reduced growth displayed by the Nc-Spain1H isolate *in vitro* and attenuated virulence in mice and bovines (Regidor-Cerrillo *et al.*, 2011; Rojo-Montejo *et al.*, 2009a; Rojo-Montejo *et al.*, 2009b). Similarly, up-regulation of NcNTPase, NcMIC1, NcROP40, and G6PD may be implicated in the high *in vitro* tachyzoite yield and pathogenicity showed by Nc-Spain7 and Nc-Liv isolates (Regidor-Cerrillo *et al.*, 2011; Regidor-Cerrillo *et al.*, 2010a; Gibney *et al.*, 2008; Williams *et al.*, 2000). Additionally, protein spots determined significantly abundant in the Nc-Liv isolate when compared with the Nc-Spain1H were also more abundant for the Nc-Spain7 isolate (Supplementary Table 1). However, identification of the up-regulated spots for Nc-Liv and Nc-Spain7 isolates was not achieved, and if they likely contribute in pathogenicity and biological characteristics displayed by Nc-Liv and Nc-Spain7 isolates, this could not be established in this study.

However, we must also consider that multiple contributions of different elements may lead to similar phenotypic traits. Recent studies of proteome comparisons in *T. gondii* have established that different protein elements and cellular processes could be implicated in the pathogenicity displayed by two type I isolates. Thus, greater expression of TgGRA 2, 3, and 6 and UPRTase in KI-1 tachyzoites and, greater expression of TgGRA 7, SAG 1, NTPases and chorismate synthase for the RH strain could both participate in the virulence of these type I strains (Choi *et al.*, 2010).

On the other hand, hydrophobic proteins such as transmembrane or membrane-associated proteins are poorly resolved using the 2-DE technique. Thus, variations in abundance of hydrophobic and membrane-associated proteins could not be assessed in this study. These elements include surface SRS-family proteins which are attached to the outer membrane of the parasite via a carboxyl-terminal glycosylphosphatidylinositol (GPI)-anchor. SRS-proteins mediate host cell attachment and invasion by the parasite, and comprise recognised tachyzoite immunodominant antigens (NcSAG1 and NcSRS2) (Jung *et al.*, 2004; Howe & Sibley, 1999). Furthermore, an arginine-rich amphipathic helix (RAH) domain has been identified in the amine-terminal half of different

proteins belonging to the *T. gondii* ROP2 kinase family. The RAH domain triggers the association of ROP2 proteins with parasitophorous vacuole membrane. Furthermore, the RAH domain is present in the virulence factors TgROP5 and TgROP18 (Reese & Boothroyd, 2009). Currently, RAH domain has not been described in *N. caninum* rhoptry kinases. In this study, blast searches in ToxoDB Database with the RAH domain described for TgROP5 showed a high percentage of identity (> 50%) with arginine-rich regions in the amine-terminal half of predicted NcROP kinase proteins (data not shown). Variations in the expression of these proteins may also contribute to phenotypic differences displayed by these three *N. caninum* isolates.

This study represents the first approach toward quantifying variations in the tachyzoite proteome of different *N. caninum* isolates, as measured by DIGE. Identification of proteins differentially abundant in the tachyzoite stage of these isolates will be valuable for obtaining a comprehensive understanding on several aspects of *N. caninum* tachyzoite biology and intra-specific diversity. This may also lead to identification of proteins involved in pathogenicity. Notably, variations in expression levels and modulation of proteins involved in invasion and proliferation processes between *N. caninum* isolates have been demonstrated. These variations could contribute to the varying *in vitro* behaviour and pathogenicity exhibited by these *N. caninum* isolates. Several proteins identified in the present study have been proposed as therapeutic targets against *Plasmodium falciparum* (Bour *et al.*, 2009), and some has been assayed as vaccine candidate against *N. caninum* infection (Alaeddine *et al.*, 2005). Further larger-scale experiments that include a higher number of representative *N. caninum* natural population isolates and use DIGE combined with other complementary proteomic platforms, such as gel-liquid chromatography coupled tandem mass spectrometry and MudPIT, could help us to identify new elements implicated in *N. caninum* pathogenicity. Eventually, this could lead to identification of new targets for new therapeutic measures against neosporosis.

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Role of the funding source

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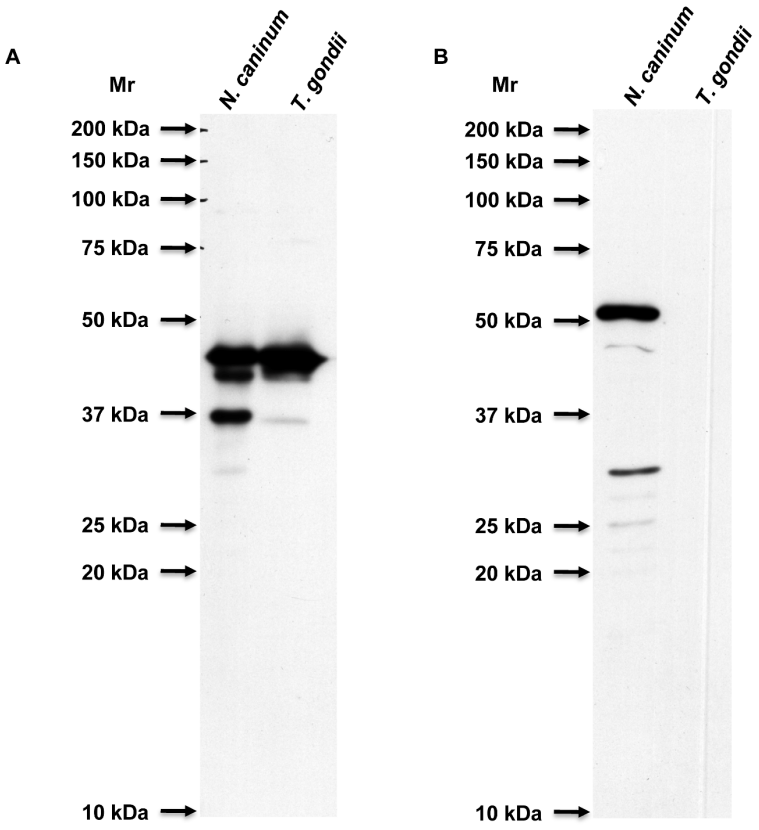
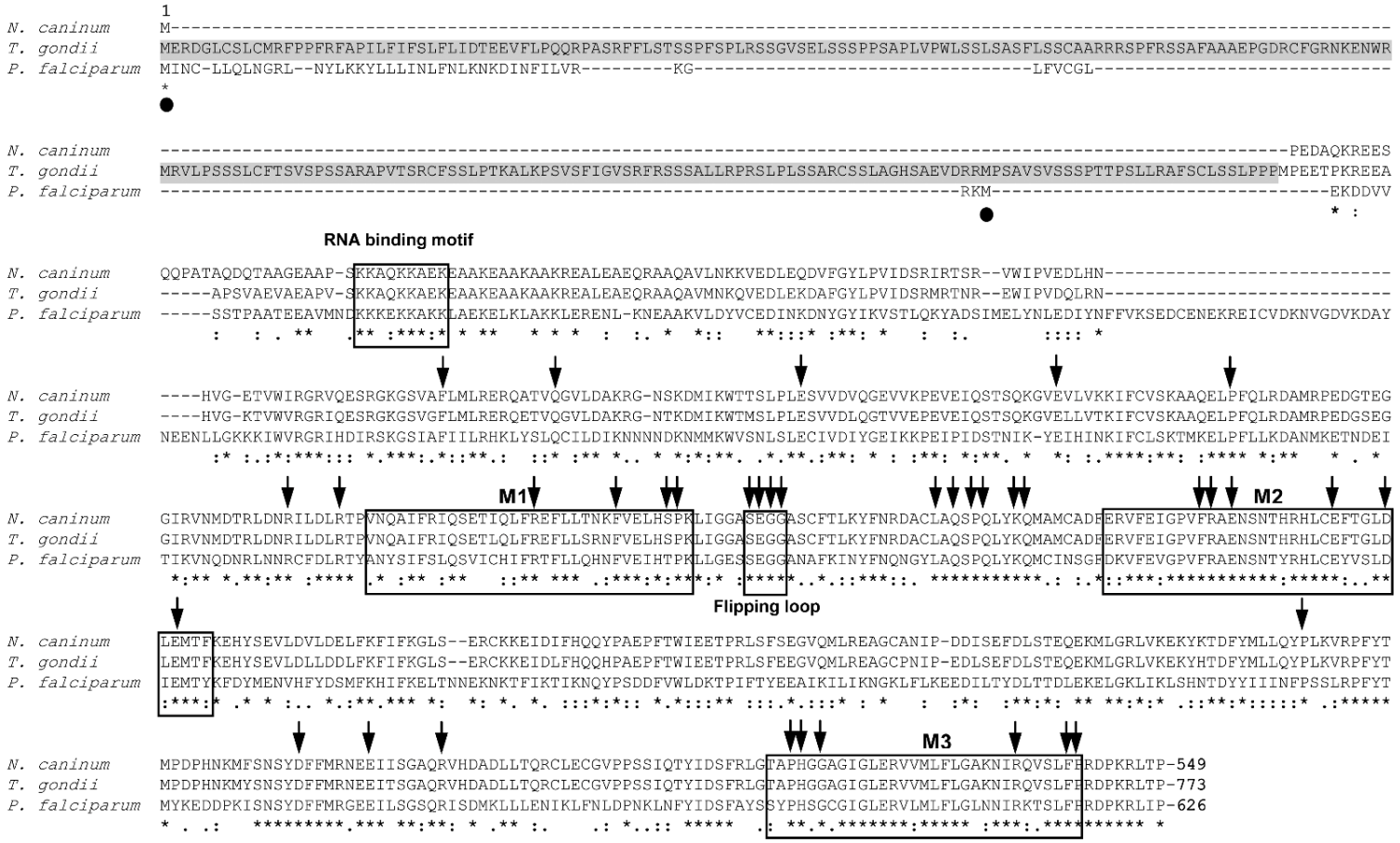
Author Contributions

Conceived and designed the experiments: JRC, GAG, LMOM. Performed the experiments: JRC, GAG, IPF, VMH, MGB, LMOM. Analyzed the data: JRC, GAG, IPF, LMOM. Wrote the paper: JRC, GAG, MGB, LMOM.



Supplementary Figure 1: Western blot analysis of Nc-Liv isolate *N. caninum* and ME49 strain *T. gondii* lysates (equivalent to $2x10^7$ tachyzoites) with a rabbit polyclonal anti-serum against actin of *T. gondii* (A) and against rNcROP40 protein (B). Note that anti-actin antibodies recognize 44 kDa and 36.5 kDa bands in *N. caninum* and *T. gondii* extracts, whereas anti-rNcROP40 antibody label 53 kDa and 29 kDa bands solely in *N. caninum* extract. Mr, molecular weight markers.



Supplementary Figure 2: Sequence alignment of *N. caninum*, *T. gondii* and *P. falciparum* AspRSs by COBALT software (ftp://ftp.ncbi.nlm.nih.gov/pub/agarwala/cobalt). Sequences are from PlasmoDB: *Plasmodium falciparum* 3D7 (PFA0145c) and from ToxoDB: *N. caninum* Nc-Liv (NCLIV_022400) and *T. gondii* ME49 (TGME49_002530). Characteristic class II motifs (M1-M3) and flipping loop for AspRSs are boxed. Arrows indicate aminoacids that are strictly conserved in all known AspRS, as previously reported (Bour *et al.*, 2009). N-terminal extension in the *T. gondii* AspRS is highlighted by a grey background. Dots indicate the two alternative methionine residues coded by in-frame ATG initiation codons in *P. falciparum* (Bour *et al.*, 2009).

Supplementary Table 1: Summary of differentially abundant spots determined by 2D-DIGE analyses from three *Neospora caninum* isolates.

Spot no.	ANOVA *P*-value	Nc-Spain7 *vs.* Nc-Spain1H comparison		Nc-Liv *vs.* Nc-Spain1H comparison		Nc-Spain7 *vs.* Nc-Liv comparison	
		Average ratio	t-test *P*-value*	Average ratio	t-test *P*-value*	Average ratio	t-test *P*-value*
251	0.033	1.79	0.033*	1.17	0.4	1.53	**0.012***
253	0.043	1.78	0.041*	1.23	0.31	1.45	**0.015***
289	0.00051	2.02	0.0019*	1.34	0.02	1.51	**0.0079***
290	0.024	2.6	0.029*	1.59	0.071	1.64	**0.15**
291	0.0038	1.94	0.0033*	1.58	0.028*	1.23	**0.17**
294	0.0049	2.09	0.0095*	1.36	0.083	1.54	**0.019***
440	0.013	2.16	0.026*	1.08	0.52	2	**0.0081***
441	0.00047	2.38	0.00065*	1.2	0.31	1.99	**0.0026***
443	0.0011	3.14	0.0035*	1.91	0.021*	1.64	**0.012***
478	0.034	1.94	0.032*	1.76	0.012*	1.1	**0.82**
506	0.02	1.9	0.021*	1.69	0.062	1.12	**0.41**
520	0.034	2.17	0.013*	1.74	0.056	1.25	**0.39**
521	0.026	2.21	0.026*	1.26	0.17	1.76	**0.095**
692	0.044	1.98	0.044*	1.25	0.11	1.59	**0.15**
698	0.0022	1.61	0.01*	-1.05	0.81	1.69	**0.00036***
700	0.0024	2.15	0.0015*	1.39	0.051	1.55	**0.05**
709	0.037	2.05	0.018*	1.28	0.35	1.6	**0.098**
711	0.0042	2.03	0.0066*	1.14	0.4	1.77	**0.0087***
768	0.019	1.32	0.083	-1.11	0.29	1.47	**0.011***
775	0.036	1.66	0.022*	1.1	0.59	1.5	**0.081**
779	0.0065	1.64	0.0047*	1.28	0.025	1.28	**0.13**
828	0.029	1.76	0.0035*	1.17	0.57	1.5	**0.074**
899	2.70E-05	6.7	2.80E-05*	1.41	0.28	4.74	**0.00038***
923	0.0032	-2.13	0.0076*	1	0.99	-2.14	**0.0099***
934	0.025	1.92	0.037*	1.13	0.28	1.69	**0.06**
939	0.0034	1.67	0.0076*	1.27	0.079	1.31	**0.0042**
957	0.0095	-2.21	0.017*	1.03	0.97	-2.27	**0.0065***
1006	0.015	1.57	0.013*	1.11	0.34	1.41	**0.053**
1036	0.02	1.53	0.012*	1.22	0.19	1.25	**0.071**
1069	0.0068	1.55	0.00025*	1.15	0.34	1.35	**0.047**
1079	0.017	1.7	0.011*	1.07	0.73	1.58	**0.041***
1084	0.0039	1.62	0.007*	1.22	0.046	1.33	**0.038**
1101	0.029	-1.38	0.045	1.16	0.35	-1.59	**0.037***
1126	0.0051	2.06	0.0024*	1.07	0.84	1.93	**0.014***
1158	0.049	1.5	0.024*	1.03	0.91	1.47	**0.032***
1202	0.0052	-1.41	0.0015*	-1.09	0.35	-1.3	**0.024**
1215	0.00028	-4.67	0.00056*	1.1	0.8	-5.16	**0.0012***
1227	6.60E-07	2.93	2.10E-05*	-1.05	0.55	3.08	**2.10E-05***
1306	0.0029	-1.79	0.0037*	-1.15	0.31	-1.56	**0.011***
1312	0.0003	-1.95	0.00072*	-1.25	0.045	-1.56	**0.0061***
1372	0.028	1.37	0.0027	-1.06	0.63	1.45	**0.035***
1385	0.0054	-1.5	0.0091*	-1.26	0.033	-1.19	**0.074**
1419	0.042	-1.96	0.0083*	-1.4	0.19	-1.39	**0.25**
1420	0.006	-1.45	0.0021*	-1.31	0.022	-1.1	**0.39**
1510	0.00021	-1.61	0.00083*	-1.18	0.082	-1.36	**0.00046**
1618	0.0077	1.96	0.017*	-1.07	0.94	2.1	**0.0018***
1659	0.0047	1.78	0.014*	-1.26	0.37	2.24	**0.0023***
1681	0.044	1.51	0.0094*	1.07	0.76	1.42	**0.056**
1715	0.0087	1.89	0.011*	1.28	0.13	1.48	**0.033***
1790	**0.0017**	**-2.1**	**0.003***	**-1.35**	**0.14**	**-1.56**	**0.0022***

Supplementary Table 1 (continuation): Summary of differentially abundant spots determined by 2D-DIGE analyses from three *Neospora caninum* isolates.

Spot no.	ANOVA *P*-value	Nc-Spain7 *vs.* Nc-Spain1H comparison		Nc-Liv *vs.* Nc-Spain1H comparison		Nc-Spain7 *vs.* Nc-Liv comparison	
		Average ratio	t-test *P*-value*	Average ratio	t-test *P*-value*	Average ratio	**t-test *P*-value***
1838	0.0047	1.14	0.2	-1.33	0.036	1.52	**0.0019***
1852	7.00E-07	4.32	1.10E-05*	-1.08	0.61	4.65	**1.30E-05***
1854	0.014	-1.67	0.015*	1.1	0.65	-1.83	**0.0098***
1855	0.00007	-2.59	0.00043*	-1.03	0.8	-2.52	**0.00068***
1971	0.0019	-2	0.0029*	-1.16	0.3	-1.72	**0.0089***
1977	8.40E-05	-1.8	8.50E-05*	-1.01	0.82	-1.78	**0.0012***
1988	0.00084	2.07	0.0037*	-1.05	0.83	2.18	**0.00062***
2021	0.00055	-1.58	0.0019*	-1.26	0.0066	-1.25	**0.021**
2211	0.023	-2.42	0.011*	-1.49	0.22	-1.63	**0.071**
2304	0.01	-1.58	0.0024*	-1.3	0.094	-1.21	**0.16**
2366	0.023	1.35	0.098	-1.31	0.19	1.76	**0.013***
2540	0.033	-1.43	0.018*	-1.27	0.12	-1.12	**0.27**
2563	0.0012	-3.37	0.0012*	-1.6	0.1	-2.1	**0.0094***
2622	0.0047	-1.94	0.0046*	-1.39	0.085	-1.39	**0.037**
2641	0.015	1.38	0.037*	-1.23	0.23	1.7	**0.011***
2648	**0.045**	**1.98**	**0.0059***	**1.27**	**0.53**	**1.57**	**0.099**

***** Denotes differently expressed spots according a ± 1.4-fold change and a *P* value < 0.05 for each comparison.

Suplementary Table 2: Summary of pentose phosphate shunt (PPP)-related enzymes in *N. caninum* and *T. gondii* ME49.

Enzyme	EC Nº*	*T. gondii* protein (ToxoDB)	*N. caninum* orthologue (ToxoDB)	Signal peptide prediction
Glucose 6-P dehydrogenase I[&]	1.1.1.49 3.1.1.31[&]	TGME49_078830[&]	NCLIV_067180	No
Glucose 6-P dehydrogenase II	1.1.1.49	TGME49_094200	NCLIV_000940[§]	Yes
6-phosphogluconate dehydrogenase I	1.1.1.44	TGME49_107850	NCLIV_060600§	No
6-phosphogluconate dehydrogenase II	1.1.1.44	TGME49_042600	NCLIV_017630	Yes
Ribulose 5-phosphate 3-epimerase	5.1.3.1	TGME49_047670	NCLIV_065340	No
Ribose 5-phosphate isomerase	5.3.1.6	TGME49_039310	NCLIV_015970	No
Transketolase	2.2.1.1	TGME49_118310	NCLIV_011210	No
Transaldolase	**2.2.1.2**	**TGME49_029360**	**NCLIV_030290**	No

* EC (Enzyme Commission) number has been provided by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.

[&] Predicted to operate with 6-phosphogluconolactonase activity.

[§] Enzymes differentially abundant or modulated across Nc-Liv, Nc-Spain1H and Nc-Spain7 isolates.

Neospora caninum tachyzoite immunome study reveals differences among three biologically different isolates

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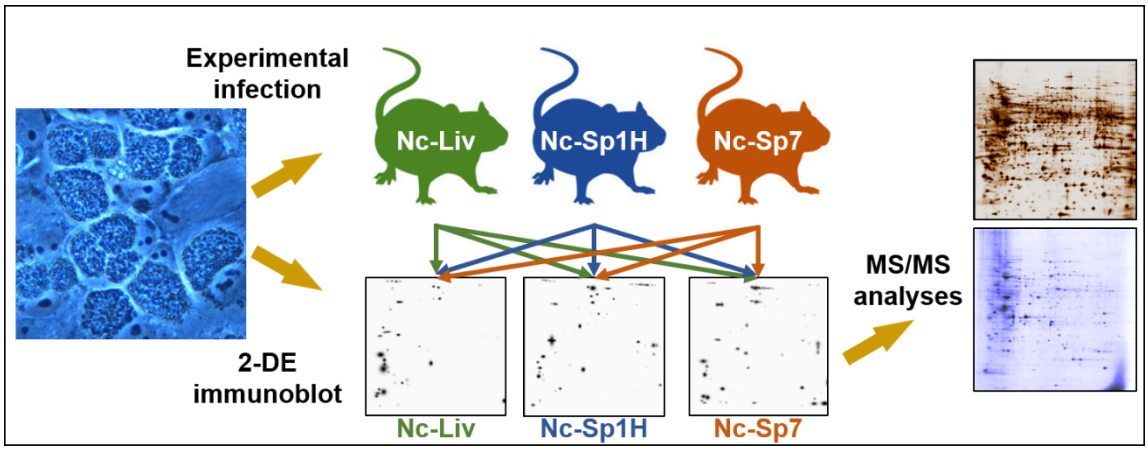

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ABSTRACT

Pathogenesis of bovine neosporosis is determined by different host- and parasite-dependent factors, including isolate virulence. A previous study identified that several *Neospora caninum* tachyzoite proteins were more abundant in virulent isolates, Nc-Liv and Nc-Spain7, compared with the low-virulent isolate Nc-Spain1H. Herein, we explored differences in the immunomes of these three isolates. Protein extracts from the Nc-Liv, Nc-Spain1H and Nc-Spain7 isolates were analysed in a 3x3 design by 2-DE immunoblot using sera from experimentally infected mice with these same three isolates. All isolates displayed a highly similar antigenic pattern when they were assessed using the same serum. Most of the reactive spots were located in the acidic region (pH 3-7) and grouped in 3 antigenic areas (250-70, 45-37 and 35-15 KDa). Differences found in the immunome depended on the sera used, regardless of the extract employed. In this sense, sera from Nc-Liv and Nc-Spain7 infected mice recognized the highest number of antigens, followed by Nc-Spain1H infected mice sera. In fact, 4 proteins identified by MS were not consistently detected in each isolate extract by sera from low-virulent Nc-Spain1H-infected mice: serine-threonine phosphatase 2C and superoxide dismutase (related to metabolism), gliding associated protein GAP45 (related to tachyzoites invasion), and NcGRA1 (located on dense granules). Similarly, 4 non-identified spots and another 2 spots chains located in 45-37 kDa area were not detected by this pooled sera. Variations between virulent Nc-Spain7 and Nc-Liv were limited to the absence of recognition by sera from Nc-Spain7-infected mice of GAP45 and the spot chains located in the 45-37 kDa area. These results suggest that variations in the immunome profiles rely on the immune response induced by each isolate and that these differentially recognized antigens could be investigated as putative virulence markers of neosporosis.

1. Introduction

Bovine neosporosis, which is caused by the cyst-forming apicomplexan parasite *Neospora caninum,* is considered one of the main causes of bovine abortion worldwide. The currently known definitive hosts of *N. caninum* are the dog (McAllister *et al.*, 1998), coyote (Gondim *et al.*, 2004), dingo (King *et al.*, 2011) and grey wolf (Dubey *et al.*, 2011). Cattle, and a wide range of other ungulates, act as intermediate hosts (Dubey *et al.*, 2014; Dubey & Schares, 2011; Dubey *et al.*, 2007a). The life cycle of *N. caninum* comprises three distinct invasive stages: tachyzoites, bradyzoites contained in tissue cysts, and sporozoites contained in oocysts. In cattle, tachyzoites are responsible for the acute stage of the infection. They multiply rapidly and endogenously, which leads to multiple rounds of lytic cycles and tissue damage. However, once the mature immune response is established, tachyzoites become bradyzoites as an immune evasion mechanism. Bradyzoites multiply slowly in tissue cysts and are responsible for the chronic stage of the infection (Dubey *et al.*, 2006). Transplacental transmission (TT) from an infected dam to the foetus can occur in postnatally acquired infections via the ingestion of oocysts (exogenous TT) or as a consequence of the reactivation of an infection during pregnancy in a chronically infected cow (endogenous TT) (Trees & Williams, 2005).

Different host and parasite factors determine the outcome of neosporosis infection, including the TT rate during pregnancy. In this sense, the relevance of the isolate responsible for the infection in bovine neosporosis has been recently highlighted. This is due to a marked variation in the capacity of different isolates to cause foetal mortality (Chryssafidis *et al.*, 2014; Regidor-Cerrillo *et al.*, 2014; Caspe *et al.*, 2012; Rojo-Montejo *et al.*, 2009a). The pathogenicity of *N. caninum* isolates has also been widely studied in cerebral and congenital mouse models, which were used to identify significant variations in virulence (Dellarupe *et al.*, 2014a; Regidor-Cerrillo *et al.*, 2010a; Rojo-Montejo *et al.*, 2009b; Atkinson *et al.*, 1999). A potential association between the tachyzoite proliferation rates in cell cultures and their pathogenicity in pregnant mouse models has been indicated (Dellarupe *et al.*, 2014b; Regidor-Cerrillo *et al.*, 2011). However, to date, the host- and parasite-dependent mechanisms that are responsible for these differences have not been completely revealed.

Proteomic approaches were previously employed to investigate relevant parasite biological processes, the protein profiles of parasite isolates and stages, as well as parasite-species (Wastling *et al.*, 2009). Regidor-Cerrillo *et al.* (2012) used two-dimensional difference in gel electrophoresis (2D-DIGE) to compare three *N. caninum* isolates (Nc-Liv, Nc-Spain1H and Nc-Spain7) that displayed different *in vivo* and *in vitro* behaviour. This work demonstrated differences in the abundance of certain proteins that are involved in invasion and proliferative processes, including some immunodominant antigens (IDA) that could explain the variations in parasite virulence among isolates. Herein, we conducted a complementary approach to further investigate the potential influence of different isolates on the immune response pattern of experimentally infected mice. Differences in the immunome of one low virulent *N. caninum* isolate (Nc-Spain1H) and two highly virulent isolates (Nc-Liv and Nc-Spain7) were explored by two dimensional electrophoresis (2-DE) immunoblotting coupled to MS/MS analyses.

2. Materials and methods

2.1. Parasites and protein extracts

N. caninum tachyzoites from Nc-Liv, Nc-Spain1H and Nc-Spain7 isolates (Rojo-Montejo *et al.*, 2009b; Regidor-Cerrillo *et al.*, 2008; Barber *et al.*, 1995) were maintained in a MARC-145 monolayer and in DMEM media supplemented with 2% foetal bovine serum. There were a limited number of culture passages (Nc-Liv, passage 24-27; Nc-Spain1H, passage 27-30; Nc-Spain7, passage 14-17). Tachyzoites for protein extracts were recovered from cultures and purified as previously described (Regidor-Cerrillo *et al.*, 2012). For protein extraction, frozen tachyzoites (2 x 10^8 /batch) were resuspended in 200 µl of lysis buffer containing 6 M urea, 2 M thiourea, 4% (w/v) (CHAPS), 65 mM 1,4-dithioerythreitol (DTE), 10 mM Tris-HCl pH 7, and 1 mM phenylmethanesulphonyl fluoride (PMSF) (added fresh) and processed by 3 cycles of rapid freezing and thawing in liquid nitrogen. After sonication, solubilisation was aided by subsequent addition of 200 µl of rehydratation buffer containing 8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 65 mM DTE, and 1% ampholyte. Insoluble material was removed by

centrifugation at 13,000 rpm for 30 min at 4 °C, and protein concentration of the resulting supernatants was determined by the Bradford method (Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as the standard.

2.2. Sera sample selection and experimental design

Sera from BALB/c mice experimentally infected with the Nc-Liv, Nc-Spain1H and Nc-Spain7 isolates were obtained from previous studies (Pereira García-Melo *et al.*, 2010; Rojo-Montejo *et al.*, 2009b; Collantes-Fernández *et al.*, 2006b). Mice were inoculated with 10^6 tachyzoites of each isolate, and sera were collected at day 32 post infection and maintained at -80 °C until use. All mice handling procedures complied with the EU legislation and were approved by the Ethics Commitee of the Complutense University of Madrid.

For 2-DE immunoblotting, three pools of sera from mice that had been experimentally infected with Nc-Liv, Nc-Spain1H, and Nc-Spain7 isolates were obtained. Each positive pool was composed of 5 sera. Additionally, a pool of sera from 5 uninfected mice was used as a negative control for immunoblotting. Pooled sera were titrated by *N. caninum* IFAT analysis using Nc-Liv tachyzoites as the antigen (Álvarez-García *et al.*, 2003). Pooled sera from mice inoculated with Nc-Liv and Nc-Spain7 achieved titers of 1:200. The titer was 1:50 for those mice infected with the Nc-Spain1H isolate.

Tachyzoite protein extracts from the Nc-Liv, Nc-Spain1H, and Nc-Spain7 isolates were analyzed by 2DE-immunoblotting using the three mouse sera pools. A 3x3 experimental design was implemented to compare the isolate-specific antigenic profile and the induced immunogenic profiles for each isolate. In addition, protein extracts from the Nc-Liv isolate were analyzed, using the uninfected pool as a negative control.

2.3. 2-DE SDS-PAGE and immunoblot

Protein extracts from Nc-Liv, Nc-Spain1H, and Nc-Spain7 were resolved by 2DE-SDS PAGE using immobilized pH gradient (IPG) strips. For the immunoblot, 100 µg of each extract was loaded onto ReadyStrip™ IPG Strips, pH 3–10 NL, (17 cm, Bio-Rad) and transferred to PVDF membranes as described below. Three replicates were carried out for each isolate and for each sera pool employed in the study. Coomasie Brilliant Blue (CBB) (Kang, D., Gho, S.G., Suh, M., Kang, C., 2002), and silver stained gels were obtained for the Nc-Liv isolate, as described for immunoblotting, but we loaded 400 and 100 µg of protein extracts, respectively. To obtain the highest resolution of the antigenic spots identified in the acidic region, replicates for the Nc-Liv protein extract were performed for immunoblot and CBB staining on ReadyStrip™ IPG Strips pH 3–6 L (17 cm, Bio-Rad).

Isoelectric focusing (IEF) of tachyzoite protein extracts was performed by passive rehydration for 16 h at 20 °C using a Protean IEF cell system (Bio-Rad). The first dimension was carried out using a program of 500 V for 2 h, 500–2000 V for 2 h, 2000–4500 V for 6 h, and 4500 V for 12 h. Strips were either processed immediately for second-dimension electrophoresis or stored at −80 °C until use. Before the second-dimension separation, proteins on the strips were reduced with 4% DTE and then alkylated with 5% iodoacetamide in equilibration buffer (6 M urea,

50 mM Tris–HCl pH 6.5, 30% glycerol, and 2% SDS). Second-dimension electrophoresis and immunoblot were performed following a previously described method (García-Lunar *et al.*, 2013). After 2-DE SDS–PAGE separation, 3–10 NL and 3–6 L IPG strip gels were transferred using transfer Tris buffer at 18 °C onto PVDF membranes for immunoblot. The blotted membranes were blocked with TBS-Tween 20 buffer that contained 5% (w/v) dry milk and were then incubated with pooled mice sera. IFAT titers for each pooled sera were employed to adjust the IgG antibody levels that were involved in immunoblot. Thus, Nc-Liv and Nc-Spain7 pooled sera were diluted at 1:800, whereas Nc-Spain1H and uninfected pooled sera were diluted at 1:200. Subsequently, the membranes were incubated with an anti-murine IgG antibody conjugated with peroxidase (Sigma-Aldrich, St. Louis, MO, USA) at a 1:10,000 dilution. Membranes were exposed for 1–5 min using the Immobilon Western Chemi-luminescent HRP Substrate, a chemiluminescence method (Millipore, Billerica, MA, USA).

2.4. Image capture and matching

All of the spots detected by the PDQuest 2-D Analysis Software (Bio-Rad) were manually verified, and false-positive spots (artefacts) were manually removed from the images. To compare the spots, a MatchSet was created from the images of the gels and immunoblots as previously described (García-Lunar *et al.*, 2013). When a spot was present in at least two immunoblot replicates, it was considered to be consistently recognized by mice sera. All gels and immunoblots performed in the study were highly reproducible because the match rates varied from 80 to 100%.

2.5. Protein identification by MALDI-TOF MS

Sufficiently abundant spots that could be matched to immunoreactive spots were excised from 400 µg CBB-stained gels for protein identification by MALDI-TOF MS. Silver-stained gel was used to check the location of the excised spots and verify the absence of neighbouring spots.

MS fingerprinting was performed in a MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyzer; PerSeptive Biosystems) that was operated in reflector mode with an accelerating voltage of 20,000 V. All mass spectra were calibrated externally using a standard peptide mixture (Sigma). For protein identification, monoisotopic peptide masses were compared with the National Center for Biotechnology Information non-redundant (NCBI nr) and ToxoDB release-6.0 databases using the MASCOT algorithm v2.1 (Matrix Science) through the Global Protein Server v3.5 from Applied Biosystems. The apicomplexan specific ToxoDB database contains *N. caninum* annotated protein sequences. For MS/MS sequencing analyses, suitable precursors were selected, and fragmentation was carried out using CID (atmospheric gas was used) with a 1 Kv ion reflector mode and precursor mass Windows +/- 10 Da. The plate model and default calibration were optimized for the MS-MS spectra processing. MASCOT search parameters were as follows: carbamidomethyl cystein as fixed modification and oxidized methionine as variable modification; peptide mass tolerance 50-100 ppm; 1 missed trypsin cleavage site; and

MS/MS fragment tolerance 0.3 Da. The parameters for the combined search (Peptide mass fingerprint and MS/MS spectra) were as described above. Identification was accepted when the probability scores were greater than the score fixed by MASCOT with a *p*-value < 0.05 (2-DE). The correlation of the gel region with both the predicted molecular weight and p*I* was also considered for protein identification.

3. Results and discussion

Main immunoreactive spots detected in 2-DE immunoblots are highlighted in Figure 1 and shown in Table 1. The analysis of immunoblots showed that most of the recognized spots by infected mice sera were located in the acidic range of the pH gradient 3-7 and between 250-70, 45-37, and 35-15 KDa (Figure 1). The average number of spots for all replicates, regardless of the antigen and pooled sera, was 59.2 ± 3.5. There were no spots detected with pooled sera from uninfected mice on the Nc-Liv immunoblot (data not shown). Recognized spots correlated with the 1-DE antigenic pattern of the tachyzoite stage of *N. caninum*. This was previously described for diagnostic purposes, where 4 IDAs (17-18, 34-35, 37 and 60-62 kDa) were identified using sera from *N. caninum* naturally infected cattle (Álvarez-García *et al.*, 2002). In addition, the same pattern was recognized in 1-DE western blot by sera from bovine or mice experimentally infected with the Nc-Spain1H isolate (Rojo-Montejo *et al.*, 2009b). Similar IDAs, including 17, 29/30, 37 and 46 kDa, were observed from the sera of experimentally infected cattle, dogs, sheep, goats, rabbits, and pigs (Bjerkås *et al.*, 1994). Notably, similar 2-DE immunomes were described for *N. caninum* tachyzoite extracts with sera from experimentally infected rabbits and cattle in previous studies (Lee *et al.*, 2005; Shin *et al.*, 2005b; Shin *et al.*, 2004; Lee *et al.*, 2003), which suggests the conservation of major IDAs among natural and experimentally infected hosts.

Comparison analyses showed that the three isolate-specific extracts employed in this study presented very similar antigenic profiles using the same pooled sera (Figure 1, Table 1). Only spot D was not recognized by sera from Nc-Spain7 infected mice in any replicate of the Nc-Liv extract, and it was very weakly recognized in the Nc-Spain1H extract. On the contrary, this spot was consistently detected in the Nc-Spain7 extract replicates. Similarly, spot 5 was weakly detected in the Nc-Liv and Nc-Spain1H extracts, whereas it was strongly immunoreactive in the Nc-Spain7 extracts by sera from Nc-Spain1H-infected mice (Figure 1, Table 1). These findings suggest minor differences in antigenic composition among the Nc-Liv, Nc-Spain1H, and Nc-Spain7 protein extracts. In previous studies, the KBA2 and JPA1 isolates exhibited almost identical 2-DE patterns when their proteomes and immunomes were compared by employing rabbit anti-*N. caninum* KBA2 serum (Lee *et al.*, 2005). Shin *et al.* (2005) also described similar 2-DE proteomes and immunomes between KBA2 and VMDL-1 isolates using bovine anti-*N. caninum* KBA2 serum. However, a limited number of isolate-specific spots were detected in KBA2 and VMDL-1 proteomes (17 and 6 spots, respectively), and specific immunoreactive spots were recognized either in KBA2 extracts or VMDL-1 extracts (8 and 1 spots, respectively). The three *N. caninum* isolate

immunomes compared in this study were very similar. These results agreed with previous findings observed by Regidor-Cerrillo *et al.* (2012), where isolate-specific spots could not be detected in the proteome by 2D-DIGE, although significant variations in protein abundance were detected among isolates. Therefore, it was expected to find non-substantial variations in the antigenic profile for these isolates in the present work. Moreover, a total representation of antigens under the conditions used in this study was ensured, i.e., the quantity of protein loaded in the gels.

Interestingly, the major differences observed in this study uniquely depended on the pool of sera used and, consequently, on the IgG responses induced in mice based on the recognition of a variable number of antigens. Seven immunogenic spots (identified by no. 8, 10, 15, 16, A, D and E in Figure 1 and Table 1) were detected differently according to the pooled mice sera employed. Two additional immunogenic spot chains (identified in area B in Figure 1 and Table 1), located in the region of 45-37 kDa, were also differently recognized by mice sera. Notably, the sera from mice experimentally infected with Nc-Liv showed the most complete recognition pattern because all of the immunoreactive spots were strongly and consistently detected, regardless of each isolate-specific antigen extract. In particular, spot no. 8 and spot chains in area B were not recognized in any replicate by the Nc-Spain7 pool sera (Figure 1, Table 1). Furthermore, the lowest spot recognition was achieved when sera from mice infected by Nc-Spain1H was assayed because a total of 7 spots (spot no. 8, 10, 15, 16, A, D and E) were not detected or were only sporadically and very weakly recognized. The recognition of spot chains in area B by Nc-Spain1H sera was also limited to the sporadic detection of a unique spot (Figure 1, Table 1). No previous studies have reported differences in the immunogenic pattern induced in different isolates from *N. caninum* or *T. gondii*. Interestingly, marked differences in antigenic profiles could not be determined when sera from rabbits immunized with tachyzoite extracts of the closely related species *B. besnoiti* and *B. tarandi* were compared by 2-DE immunoblots (García-Lunar *et al.*, 2014).

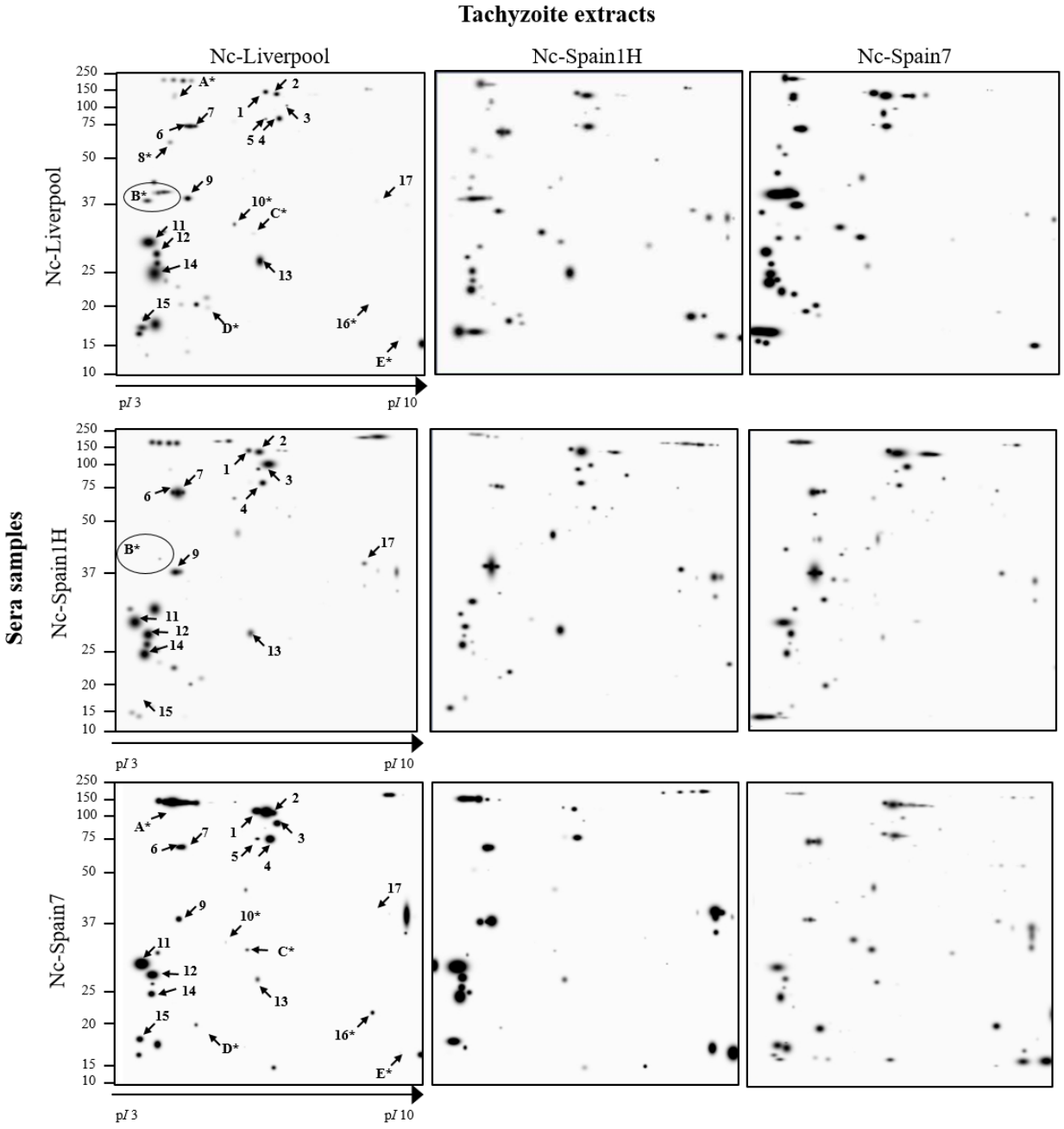

Figure 1: Immunome of the tachyzoite stage of Nc-Liv, Nc-Spain1H, and Nc-Spain7 isolates of *N. caninum*. The master image of each tachyzoite extract and mouse serum generated from three triplicates for each condition is shown. The protein extract is indicated on top, and the serum employed is shown on the left-hand side. Note that master gels are a representation of three replicates, where only the spots represented in the three replicates are represented (Table 1). Protein spots that were identified by MS/MS are marked with arrows and numbered. Additional protein spots that were differentially recognized by pooled mice sera are marked with arrows and identified by letters. Spot chains in area B are highlighted with a circle. Asterisks marks all differentially recognized protein spots.

Table 1: Immunoreactivity of spots on tachyzoite protein extracts from Nc-Liv, Nc-Spain1H, and Nc-Spain7 isolates by sera from experimentally infected mice using the same three isolates according to a 3x3 experimental design.

Spot[a]	Identified protein	Nc-Liv sera[b]			Nc-Spain1H sera[b]			Nc-Spain7 sera[b]		
		Liv extract	Sp1H extract	Sp7 extract	Liv extract	Sp1H extract	Sp7 extract	Liv extract	Sp1H extract	Sp7 extract
1	Subtilisin-like serine protease	+++	+++	+++	+++	+++	+++	+++	+++	+++
2	Subtilisin-like serine protease	+++	+++	+++	+++	+++	+++	+++	+++	+++
3	Subtilisin-like serine protease	+++	+++	+++	++	+++	+++	+++	+++	+++
4	Subtilisin-like serine protease	+++	+++	+++	+++	+++	+++	+++	+++	+++
5	Subtilisin-like serine protease	+++	+++	+++	+	+	+++	+++	+++	+++
6	Heat shock protein 70, putative	+++	+++	+++	+++	+++	+++	+++	+++	+++
7	Heat shock protein 70, putative	+++	+++	+++	+++	+++	+++	+++	+++	+++
8	Gliding-associated protein 45	++	++	+++			+			
9	Actin	+++	+++	+++	++	+++	+++	++	+++	+++
10	Serine-threonine phosphatase 2C	+++	+++	+++	+			+++	++	++
11	GRA7	+++	+++	+++	+++	+++	+++	+++	+++	+++
12	Myosin light chain	+++	+++	+++	+++	+++	+++	+++	+++	+++
13	GRA2 protein	+++	+++	+++	+++	+++	+++	+++	+++	+
14	Tubuline beta chain	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Surface protein rhoptry, putative	+++	+++	+++	+++	+++	+++	+++	+++	+++
15	GRA1	++	++	+++	+			+++	+++	+++
16	Superoxide dismutase	+++	+++	+++				+++	++	+++
17	Fructose-1,6-bisphosphate aldolase	++	+++	++	++	+++	+++	++	+++	++
A		+	++	++				+	+	+
B		+++	+++	+++		+	+			
C		++	++	+++	+		+	+++	+++	+++
D		++	++	++	+				+	++
E		++	+++	++				++	+++	+++

Whether the variations in the pattern of recognized antigens by mice sera are dependent on the potential epitope polymorphisms among *N. caninum* isolates remains unclear. Previous studies comparing protein sequences that code for antigens, including the IDA NcGRA7, have revealed the high conservation of sequence identity among diverse isolates (for a review, see (Beck *et al.*, 2009)). Sequencing of the differentially recognized antigens described in this study could help discard the influence of epitope variations on the pattern of antigens recognized by sera. Differences due to post-translational modifications, such as acetylation, methylation, and phosphorylation patterns among isolates, may also influence the antigen recognition and immunome pattern. However, significant variations related to the source of the antigen subjected to homologous and heterologous immunoblotting were not detected, which suggested epitope conservation across these *N. caninum* isolates.

Most of the immunoreactive spots, particularly those that were differentially recognized by mice sera, were obtained from the CBB Nc-Liv gels and identified by MALDI TOF/TOF MS analysis. A total of 17 immunoreactive spots were identified using mass fingerprinting and MS/MS combined with MS. These spots are highlighted by numbers in the gels shown in Figure 1 and Figure 2 and in Tables 1 and 2. The identified 17 spots corresponded to 13 different proteins, which indicated that these protein species may involve multiple isoforms or post-translationally modified protein forms. In addition, spot no. 14 showed double protein identification (Table 2; Figure 2). Of those differently recognized spots, a total of four spots were successfully identified (no. 8, 10, 15, 16).

Common immunoreactive spots corresponded to proteins involved in metabolic pathways, tachyzoite motility, and organelle elements of apicomplexan involved in the lytic cycle, which have been commonly described in previous *N. caninum* proteomic studies. They have also already been described as antigens in several works that used sera from experimentally infected mice, rabbits, or bovines, thus confirming their antigenicity among diverse natural and experimental host species (Zhang *et al.*, 2011; Lee *et al.*, 2005; Shin *et al.*, 2005b; Shin *et al.*, 2004; Lee *et al.*, 2003) (Table 2).

Table 2: List of immunogenic antigens of the *N. caninum* tachyzoite stage identified by MS and/or MS/MS.

	Identified protein	Score	Theo. p//Mw (kDa)	Sequence coverage[a]	No. matched peptides or amino acid sequence of peptides [b, c]	Accesion number	Functional category [d, e, f, g]
1	Subtilisin like serine protease (NcSUB1)[j]	125	5.98/64	26	15/30 [b]	NCLIV 081630	Proteolisis [d]
2		162		32	25/65 [b]		
3		107		27	17/65 [b]		
4		71		22	14/65 [b]		
5		70		24	14/65 [b]		
6	HSP70[j, k]	269	5.15/73	48	33/65 [b]	NCLIV 131890	Stress response [g]
7	HSP70[j, k]	188	5.07/73	39	26/65 [b]	NCLIV 102920	Stress response [g]
8[h]	Gliding-associated P45	84	4.91/28.4	34	K.QAAEAEAAEAQRR.Q (8) [c] K.VAEHSSAAATDR.S (40) [c]	NCLIV 123060	Inner Membrane Complex [f]
9[h]	Actin[j, k]	292	5.05/42	65	33/65 [b]	NCLIV 020800	Tachyzoite motility and invasion [g]
10	Serin-threonine phosphatase 2C[j, k]	149	5.35/35	56	25/65 [b]	NCLIV 101390	Catalytic activity [e]
11[h]	GRA7[j, k]	114	4.57/22	70	10/65 [b]	NCLIV 082090	Dense granule protein [g]
12[h]	Myosin light chain	92	4.55/24.9	42	15/65 [b]	NCLIV 000030	Tachyzoite motility and invasion [g]
13	GRA2[j]	92	7.82/23.7	38	11/65 [b]	NCLIV 120660	Dense granule protein [g]
14mix[i]	Tubuline beta chain[j, k]	86	4.7/50.5	29	13/49 [b]	NCLIV 110530	Microtubule-based process [g]
	Suface rhoptry protein, putative	58	5.8/38.8	24	7/49 [b]	NCLIV 130570	Rhoptry protein (TgROP1)[g]
15[h]	GRA1[k]	341	4.39/19.84	42	R.GLSVGQSVDLDLMGR.R (8) [c] R.SEGAPDVLEISVLDADGK.A (97) [c] K.ASHIGFVSIPEVMDTVAR.M (105) [c] K.ASHIGFVSIPEVMDTVAR.M (50) [c] K.LAGDLEALQGQH (85) [c]	NCLIV 104790	Dense granule protein [g]
16	Superoxide dismutase	176	6.5/22.74	63	17/65 [b]	NCLIV 134580	Oxidation-Reduction Process [d]
17	Fructose-1,6-bisphosphate aldolase[j, k]	65	7.49/90.1	24	14/65 [b]	NCLIV 124440	Glycolitic enzyme [g]

This table was made following the MIAPE guidelines developed by HUPO-Proteomics Standard Initiative.

[a] Amino acid sequence coverage for the proteins identified by MS and MS/MS.

[b] Number of peptide mass values matched/search

[c] Amino acid sequence identified by MS/MS; the ion score is indicated in parentheses.

[d] Biological function (GO term)

[e] Molecular function (GO term)

[f] Cellular component (GO term)

[g] Function or cellular component described in previous studies

[h] Protein identification was confirmed in pH 3-6 gels by MS

[j, k] Immunogenic spots recognized by *N. caninum* experimentally infected mice and cattle sera identified in previous studies: [j] Shin *et al*., 2005 and [k] Zhang *et al*., 2011

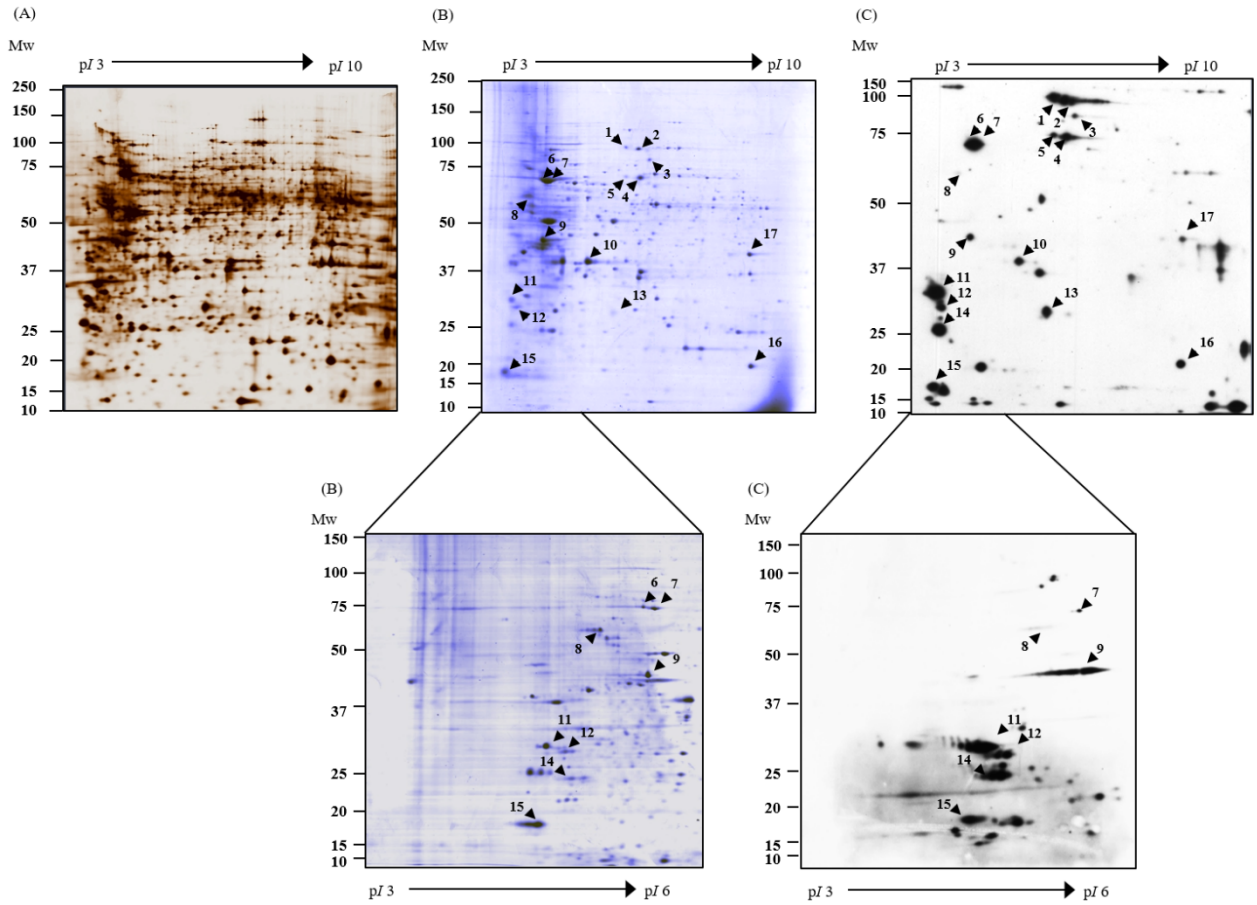

Figure 2: 2-DE gels and immunome of *N. caninum*. Silver stained (A), CBB stained (B) and immunoblot (C) of the tachyzoite stage of Nc-Liv analysed with mice sera from experimentally Nc-Liv infected mice. Proteins were separated along a non-linear pH gradient (pH 3–10 NL IPG strips; 17 cm), a linear pH gradient (pH 3–6 NL IPG strips; 17 cm) in the first dimension, and on a 10% polyacrylamide gel in the second dimension. Gels were either CBB or silver stained or transferred to PVDF membranes for immunoblot. Proteins that were identified by MS analysis are marked with arrows and numbered.

Fructose 1-6 bisphosphate aldolase and HSP70 were detected in all of the immunoblots, regardless of the sera employed. Marugán-Hernández *et al.* (2010) claimed that both proteins are more abundant in *N. caninum* bradyzoites, which is likely due to the predominant anaerobic glycolysis and stress conditions necessary for bradyzoite development. Apicomplexan parasites actively invade the host cell because of their gliding motility, which depends on parasite actin, the MyoA motor complex, and microneme proteins acting as force transmitters (Egarter *et al.*, 2014). In this sense, actin, myosin, and tubulin were identified in the present work. These proteins are related to gliding motility and invasion since they integrate the glideosome, the microtubules and the apical conoid. Interestingly, myosin light chain (NcMLC1) and actin, which were previously described to be differentially abundant among these isolates (Regidor-Cerrillo *et al.*, 2012), were identified as an antigen for the first time in the

present study. Mice sera commonly recognized two other proteins that are present in organelles: the subtilisin-like serine protease, that was identified in 5 different spots, and the surface rhoptry protein, which was described as the ortholog of TgROP1 in a previous study (Marugán-Hernández *et al.*, 2011a). Thus, Marugán-Hernández *et al.* (2011) could previously detect these proteins by analyzing subcellular fractions of *N. caninum* tachyzoites that were enriched in rhoptry elements. Finally, dense granule proteins, NcGRA2 and NcGRA7, which were secreted during and after invasion into the parasitophorous vacuole (Aguado-Martínez *et al.*, 2010; Vonlaufen *et al.*, 2004), were successfully identified in all immunoblots, regardless of the sera or antigen employed (Tables 1, 2). NcGRA2 seems to be a typically abundant antigen in *N. caninum* and is recognized by infected cow sera (Lee *et al.*, 2005; Shin *et al.*, 2005b). The NcGRA7 has been described as a major IDA for bovine neosporosis (Álvarez-García *et al.*, 2007) and has been proposed as an abortion marker in pregnant cattle (Huang *et al.*, 2007).

Differentially recognized antigens were also related to the tachyzoite motility, invasion, and organelle elements of apicomplexan. The GAP45 protein was only detected by Nc-Liv pooled sera. Interestingly, *T. gondii* gliding associated proteins 45 – 50 (TgGAP45-50) anchor the actin/myosin-based motor complex, which is formed by other identified antigens (TgActin and TgMLC1), to the inner membrane complex of the parasite (Egarter *et al.*, 2014). NcGAP45 immunoreactivity was first described in this study. The serine-threonine phosphatase 2C and superoxide dismutase, as well as NcGRA1, failed to be consistently detected by Nc-Spain1H pooled sera. Proteases and phosphatases have also been described as conserved proteins in Apicomplexan parasites that regulate a wide variety of cellular functions in the host cell (Li *et al.*, 2012). As an example, it has been recently demonstrated that protein phosphatase-2C is released by rhoptries in *T. gondii* and are targeted to the host nucleus during invasion, thus providing another way for the parasite to interact with its host cell during the invasion process (Gilbert *et al.*, 2007). Regarding superoxide dismutase, it is involved in a defence mechanism that helps protect *T. gondii* and *N. caninum* from oxygen toxicity in both tachyzoites and bradyzoites. Therefore, superoxide dismutase might be essential for the intracellular growth of both developmental stages. It also might play an important role in the pathogenesis of the parasite by protecting itself from oxidative killing by host effector cells (Cho *et al.*, 2004). The serine-threonine phosphatase 2C and NcGRA1 that were differentially recognized in this study have been previously described as *N. caninum* antigens in mice and cattle (Zhang *et al.*, 2011; Shin *et al.*, 2005b).

The results obtained in the present work provide additional data to previous intra-specific variability studies because differentially recognized targets might partially explain the differences in pathogenicity among isolates. The Nc-Liv and Nc-Spain7 isolates are considered highly virulent according to the results obtained in cerebral and pregnant mouse models (Regidor-Cerrillo *et al.*, 2011; Pereira García-Melo *et al.*, 2010; Regidor-Cerrillo *et al.*, 2010a; Collantes-Fernández *et al.*, 2006b) and in cattle (Regidor-Cerrillo *et al.*, 2014; Williams *et al.*, 2007;

Williams *et al.*, 2000). In contrast, Nc-Spain1H has been described as a low virulent isolate because of its inability to induce clinical signs or mortality in mice and abortion in cattle (Rojo-Montejo *et al.*, 2009a; Rojo-Montejo *et al.*, 2009b). In the present work, sera from Nc-Liv infected mice recognized the highest number of antigens, followed by sera from those infected by the Nc-Spain7 isolate. In contrast, sera against the Nc-Spain1H isolate showed the lowest antigen recognition. Therefore, a hierarchical pattern of antigen recognition is evoked. Hence, variations in antigen detection by mice sera could be a consequence of variations in the immune response induced in mice by these three

N. caninum isolates, which display differences in their infection dynamics and severity. Interestingly, in mice infected with high virulent isolates (Nc-Liv and Nc-Spain7), the parasite is apparently cleared from the lungs earlier than it is in mice infected with the low virulent Nc-Spain1H isolate during the acute phase of the infection (Pereira García-Melo *et al.*, 2010; Rojo-Montejo *et al.*, 2009b; Collantes-Fernández *et al.*, 2006b). It has been suggested that the low-to-moderate virulence isolates could be more exposed to the host immune response during acute infection. This fact may lead to a faster clearance of the parasite as the mature immune response is established. In contrast, high virulence isolates would remain actively proliferating, reach higher parasite burdens, and lead to a higher immune stimulation (Jiménez-Ruiz *et al.*, 2013b; Pereira García-Melo *et al.*, 2010). Therefore, the increment in the repertoire of recognized antigens by sera from mice infected with Nc-Liv and Nc-Spain7 isolates may be a consequence of the prolonged stimulation of the immune system. Moreover, high virulence isolates may induce the production of higher levels of specific anti-*N. caninum* IgG. In accordance with this hypothesis, mice infected with the Nc-Liv and Nc-Spain7 isolates showed higher antibody levels than did those infected with the Nc-Spain1H, as revealed by IFAT in this study. Interestingly, a previous study demonstrated that isolate virulence was clearly correlated with higher IgG levels against the NcGRA7 antigen (Jiménez-Ruiz *et al.*, 2013b).

In conclusion, this is the first proteomic approach to describe differences in the immunogenic profiles induced by three *N. caninum* isolates that showed different *in vivo* and *in vitro* behaviour. Specific protein spots, such as GAP45, serine-threonine phosphatase 2C, superoxide dismutase, and NcGRA1, were not detected by the low-virulent Nc-Spain1H pooled sera. Some of these antigens are also commonly recognized by sera from *N. caninum* infected bovines, and their use as virulence markers for neosporosis should be further investigated in cattle.

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Conflict of interest statement

The authors declare no conflicts of interest.

OBJETIVO 2/OBJECTIVE 2

Descripción y caracterización de las proteínas NcROP40 y NcNTPasa a lo largo del ciclo lítico del taquizoíto mediante abordajes *in silico* e *in vitro*

Description and characterization of the NcROP40 and NcNTPase proteins along the tachyzoite lytic cycle through *in silico* and *in vitro* approaches

Sub-objective 2.1:

Characterization of the *N. caninum* NcROP40 and NcROP2Fam-1 rhoptry proteins during the tachyzoite lytic cycle

Sub-objective 2.2:

The tandemly repeated NTPase (NTPDase) from *N. caninum* is a canonical GRA protein whose expression, secretion and phosphorylation coincides with the tachyzoite egress

Resumen

Hasta la fecha, los elementos que determinan la virulencia de *N. caninum* han sido poco estudiados y permanecen sin identificar. No obstante, mediante un abordaje proteómico, se ha reconocido a las proteínas NcROP40 y NcNTPasa como potenciales factores de virulencia del parásito. Ambas proteínas muestran diferencias en su abundancia entre los aislados de alta y baja virulencia, son exclusivas de los parásitos apicomplejos y han sido poco estudiadas hasta el momento. Por un lado, la proteína de roptrias NcROP40 se incluye dentro de la familia de quinasas y pseudoquinasas ROP2, a la que también pertenecen los factores de virulencia de *T. gondii* TgROP5, TgROP16 y TgROP18. No obstante, los miembros de esta familia han sido poco estudiados en *N. caninum*. Concretamente, NcROP2Fam-1 es la única proteína de las roptrias caracterizada hasta la fecha, habiéndose asociado al proceso de invasión. Esto sugiere que la proteína NcROP40 podría ser también un factor de virulencia en *N. caninum*. Por otro lado, la proteína de gránulos densos NcNTPasa parece ser relevante para todos los miembros de la familia Sarcocystidae. De hecho, la TgNTPasa guarda relación con la egresión y replicación activa de los taquizoítos de *T. gondii*. Además, la expresión de determinadas isoformas de la proteína TgNTPasa se ha asociado con la virulencia observada en los aislados tipo I de *T. gondii*. En este sentido, la NcNTPasa también parece estar relacionada con la virulencia del parásito, aunque hasta la fecha no se dispone de información específica sobre esta proteína.

En el presente objetivo se llevó a cabo una caracterización exhaustiva de las proteínas NcROP40 y NcNTPasa mediante abordajes *in silico* e *in vitro*. Por un lado, se analizaron en detalle sus secuencias codificantes y motivos funcionales. Por otro, se estudió su localización subcelular en el parásito. NcROP40 y NcNTPasa fueron comparadas con las proteínas NcROP2Fam-1 y NcGRA7, previamente caracterizadas, y analizadas en el contexto de las interacciones parásito-hospedador. Para ello, se realizó un estudio exhaustivo de su dinámica de expresión, secreción y fosforilación a lo largo del ciclo lítico del taquizoíto.

El análisis de la secuencia de NcROP40 demostró la presencia de un fragmento adicional en el extremo amino terminal de la proteína. Dicho fragmento no había sido predicho previamente en el banco de datos ToxoDB. Por otro lado, los estudios *in silico* determinaron que la proteína carece de dominios de asociación a la membrana de la vacuola parasitófora. De hecho, no se observó evidencia alguna de interacción de NcROP40 con dicha membrana mediante microscopía confocal a lo largo del ciclo lítico. Del mismo modo, no se pudo verificar la secreción de NcROP40. En cambio, la secreción de NcROP2Fam-1 se observó tras el contacto del parásito con la célula hospedadora, pudiendo detectarse en evacuolas y en la membrana de la vacuola parasitófora. No obstante, es posible que la proteína NcROP40 sea secretada al citosol de la célula hospedadora, donde su dilución impediría su detección mediante técnicas de inmunofluorescencia.

Por otro lado, se determinó la distribución de los genes que codifican la proteína NcNTPasa en el genoma del aislado Nc-Liv. Dichos genes se distribuyen en tres *loci* diferentes que podrían albergar hasta seis alelos distintos. En consonancia con estos hallazgos, se confirmó la detección de varias especies de la proteína NcNTPasa en dicho aislado. Además, se confirmó que las proteínas NcNTPasa y NcGRA7 son secretadas desde los gránulos densos y se asocian con la membrana de la vacuola parasitófora. Dicha secreción ocurre de forma espontánea tras la egresión del taquizoíto de la célula, independientemente de la ruta de señalización del calcio, o del contacto con la célula hospedadora, aunque en el caso de NcGRA7, depende también de la temperatura.

La cinética de expresión de ARNm fue similar para Nc*ROP40*, Nc*ROP2Fam-1*, Nc*NTPasa* y Nc*GRA7* a lo largo del ciclo lítico, observándose los mayores niveles de ARNm durante las fases de egresión y la invasión del parásito. No obstante, los niveles de ARNm de Nc*ROP40* no se vieron afectados tras inducir la egresión de forma artificial. Además, de entre todas las proteínas estudiadas, NcROP40 fue la única no fosforilada durante la egresión del parásito. Estos hallazgos indican que todas las proteínas participan en el ciclo lítico del parásito y que la función de NcROP2Fam-1, NcNTPasa y NcGRA7 podría ser más relevante durante la egresión o fases posteriores durante la invasión temprana como se determinó para NcROP2Fam-1 y NcGRA7 en estudios previos. En conjunto, estos resultados señalan a las cuatro proteínas como candidatos vacunales potenciales.

Characterization of the *Neospora caninum* NcROP40 and NcROP2Fam-1 rhoptry proteins during the tachyzoite lytic cycle

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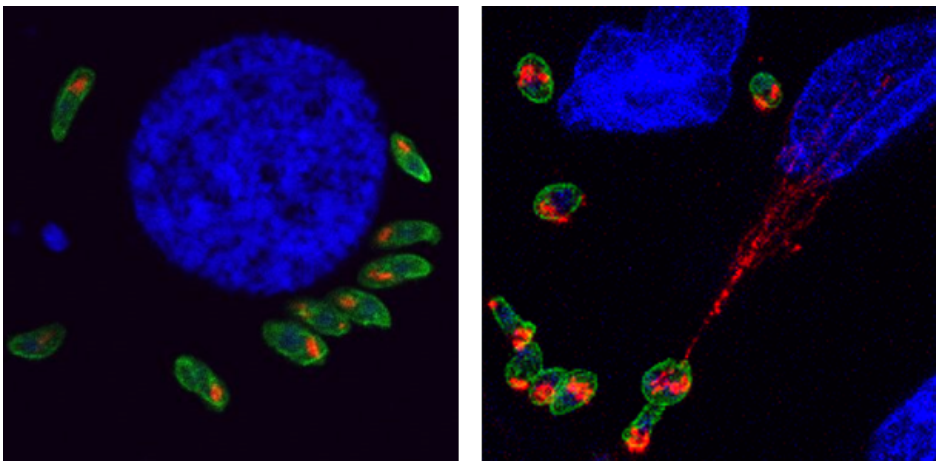

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ABSTRACT

Virulence factors from the ROP2-family have been extensively studied in *Toxoplasma gondii*, but in the closely related *Neospora caninum* only NcROP2Fam-1 has been partially characterized to date. NcROP40 is a member of this family and was found to be more abundantly expressed in virulent isolates. Both NcROP2Fam-1 and NcROP40 were evaluated as vaccine candidates and exerted a synergistic effect in terms of protection against vertical transmission in mouse models, which suggests that they may be relevant for parasite pathogenicity. NcROP40 is localized in the rhoptry bulbs of tachyzoites and bradyzoites, but in contrast to NcROP2Fam-1, the protein does not associate with the parasitophorous vacuole membrane due to the lack of arginine-rich amphipathic helix in its sequence. Similarly to Nc*ROP2Fam-1*, Nc*ROP40* mRNA levels are highly increased during tachyzoite egress and invasion. However, Nc*ROP40* up-regulation does not appear to be linked to the mechanisms triggering egress. In contrast to NcROP2Fam-1, phosphorylation of NcROP40 was not observed during egress. Besides, NcROP40 secretion into the host cell was not successfully detected by immunofluorescence techniques. These findings indicate that NcROP40 and NcROP2Fam-1 carry out different functions, and highlight the need to elucidate the role of NcROP40 within the lytic cycle and to explain its relative abundance in tachyzoites.

1. Introduction

Neospora caninum is a cyst-forming parasite that causes neuromuscular disorders in dogs, and abortion, stillbirth and birth of weak offspring in bovines. This protozoan is phylogenetically related to *Toxoplasma gondii*, with which it shares the ability to cross the placenta and to infect the foetus. In cattle, asexually proliferating tachyzoites and bradyzoites are the only stages described. Tachyzoites have a high proliferative potential and are thus responsible for the dissemination of the parasite into different tissues. Bradyzoites ensure parasite persistence by forming tissue cysts located in immune-privileged organs such as the brain (Dubey & Schares, 2011). Since these two stages are strictly intracellular, they have developed a number of mechanisms to actively invade their host cells and modulate their intracellular compartment to optimize intracellular survival and growth. These processes are grouped under the name of lytic cycle (Hemphill *et al.*, 2013). Important structures exclusively found in apicomplexans, namely the apical complex and specialized secretory organelles such as micronemes, rhoptries and dense granules play important roles in the lytic cycle. Contents of these secretory organelles are sequentially released to ensure invasion, intracellular maintenance and replication of the parasite in parasitophorous vacuoles, where they mediate and influence the host cell machinery (Kemp *et al.*, 2013). Among apicomplexan parasites, the molecular basis of the lytic cycle is highly conserved, and the underlying of mechanisms described for *T. gondii* (Carruthers & Sibley, 1997) and *Plasmodium* spp. (Cowman *et al.*, 2012) are likely to be similar in *N. caninum* (Hemphill *et al.*, 2013).

Rhoptries have been the subject of extensive studies during the last years due to the role of their proteins in host cell invasion and cell regulation processes. Some of these proteins (RONs) are restricted to the neck, and others

(ROPs) to the bulb of these organelles. RONs are involved in the formation of the moving junction required for parasite entry into the host cells (Beck *et al.*, 2014). The ROP2-family represents one of the largest and best-studied group of ROP proteins in *T. gondii*, and includes protein kinases and pseudokinases that are proven virulence factors (Etheridge *et al.*, 2014; Lei *et al.*, 2014; Reese *et al.*, 2014; Schneider *et al.*, 2013). To our knowledge, most of the ROP2-like proteins are secreted into the host cytosol during invasion and some of them can associate with the parasitophorous vacuole membrane (PVM), but their function is still largely unknown (Boothroyd & Dubremetz, 2008; Bradley & Sibley, 2007; El Hajj *et al.*, 2006). The ROP2-family has been recently catalogued in *N. caninum* (Talevich & Kannan, 2013), but only limited information is available on this protein family. Currently, only NcROP1, NcROP2Fam-1, NcROP4, NcROP5, NcROP9, NcROP30 and NcROP40 have been identified by proteomic studies (Regidor-Cerrillo *et al.*, 2012; Marugán-Hernández *et al.*, 2011a; Sohn *et al.*, 2011), but their function has not been described. To date, the only *N. caninum* rhoptry protein that has been partially characterized is NcROP2Fam-1 (Alaeddine *et al.*, 2013). This protein was previously considered the orthologue of TgROP7 (Reid *et al.*, 2012). However, it has been recently shown that TgROP7 and NcROP2Fam-1 are unlikely to be orthologues (Alaeddine *et al.*, 2013). A fragment of NcROP2Fam-1 has been employed as a vaccine in mouse models, showing relatively high protection rates against challenge infection (Debache *et al.*, 2010; Debache *et al.*, 2009; Debache *et al.*, 2008). Another rhoptry protein, NcROP40, was found to be more abundantly expressed in virulent isolates of *N. caninum* (Regidor-Cerrillo *et al.*, 2012), thus posing the obvious question whether NcROP40 plays a potential role in parasite virulence as described for other rhoptry proteins in *T. gondii*. When applied as vaccines a combined NcROP40+NcROP2Fam-1 protein formulation had a synergistic effect and was able to induce a partial block in transplacental transmission in a pregnant mouse model of neosporosis (Pastor-Fernández *et al.*, 2015a).

The aim of the present work was to characterize NcROP40 and compare its features with NcROP2Fam-1 during the lytic cycle of *N. caninum* development. This includes the molecular characterization of the NcROP40 through *in silico* studies, define its subcellular localization throughout the lytic cycle in comparison with NcROP2Fam-1, and to study protein dynamics, the transcript expression profile and their phosphorylation in order to predict their putative functional role in the different phases of the tachyzoite lytic cycle.

2. Materials and methods

2.1. In silico *analysis and Nc*ROP40 *sequencing*

All the sequences were obtained from ToxoDB v7.3. and v12 (www.toxodb.org) and edited using the BioEdit software v7.1.1. BLAST tools from NCBI (www.ncbi.nlm.nih.gov/BLAST/) and ToxoDB websites were used to match homologous sequences. Clustal Omega (www.ebi.ac.uk) was employed to align nucleotide and protein sequences. Identity and similarity percentages were calculated with the Sequence Manipulation Suite (www.bioinformatics.org). Open Reading Frames

(ORFs) and introns were predicted through the ORF Finder Tool (www.ncbi.nlm.nih.gov) and the Splign Tool (www.ncbi.nlm.nih.gov), respectively. SignalP 4.1 server (www.cbs.dtu.dk) was used to predict signal peptides. Potential alpha helices in the arginine-rich amphipathic helix (RAH) domain were searched using Jpred3 (www.compbio.dundee.ac.uk), PSIPRED v3.0 (bioinf.cs.ucl.ac.uk/ and PSSpred (zhanglab.ccmb.med.umich.edu) tools. Trans-membrane regions were predicted with the TMPred tool (www.ch.embnet.org) and protein families from Pfam database (pfam.sanger.ac.uk). Potential phosphorylation sites were analyzed by the NetPhos v2.0 (www.cbs.dtu.dk), NetPhosK v1.0 (www.cbs.dtu.dk) and the Diphos v1.3. (www.dabi.temple.edu/disphos/) servers.

The Nc*ROP40* gene (previously named Nc*ROP8*, NCLIV_012920 in ToxoDB v12) was sequenced and compared among three *N. caninum* isolates of different origins. For this purpose, total genomic DNA from Nc-Liv (Barber *et al.*, 1993), Nc-Spain7 (Regidor-Cerrillo *et al.*, 2008) and Nc-Spain1H (Rojo-Montejo *et al.*, 2009b) isolates was purified with the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's recommendations. The *NcROP40*-ORF (1176 bp) and the up and down-stream regions (750 + 992 bp) were amplified from the three isolates using the Fw-chrV_ROP40 and Rv-chrV_ROP40 primers (Supplementary Table 1). PCR conditions were 95º C for 5 min, 35 cycles at 95º C for 1 min, 58º C for 1 min and 72º C for 1 min, and a final elongation at 72º C for 10 min. PCRs were carried out with the Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) and all primers were purchased from Sigma-Aldrich. Amplified fragments were purified with the GENECLEAN Turbo kit (MP Biomedicals) from 1% low melting agarose gels. DNA was sequenced in two directions with an ABI Prism 377 DNA sequencer (Applied Biosystems) in the Genomics Unit of the Scientific Park of Madrid. Six pairs of primers were employed for this purpose (Supplementary Table 1). Sequences were edited and aligned using the BioEdit software v7.1.1.

2.2. Parasite culture

N. caninum (Nc-Liv isolate) tachyzoites were propagated *in vitro* by continuous passage in MARC-145 cell culture using standard procedures (Pérez-Zaballos *et al.*, 2005). For transmission electron microscopy, murine epidermal keratinocyte cultures were infected with the same isolate as described earlier (Vonlaufen *et al.*, 2002). *In vitro* tachyzoite-to-bradyzoite stage conversion was induced and checked by BAG1 and CC2 expression as previously described (Hemphill *et al.*, 2004). Evacuole assays were performed with the Nc-Liv isolate in human foreskin fibroblasts (HFFs) as previously described (Dunn *et al.*, 2008).

2.3. Generation of plasmids

Nc*ROP40* (NCLIV_012920 in ToxoDB v12) and Nc*ROP2Fam-1* (NCLIV_001970 in ToxoDB v12) were cloned in the pET45b(+) expression system (Novagen) as previously described (Pastor-Fernández *et al.*, 2015a; Regidor-Cerrillo *et al.*, 2012). On the other hand, Nc*Alpha Tubulin* (*TUBα*) (NCLIV_058890 in ToxoDB v12) and Nc*SAG1* (NCLIV_033230 in ToxoDB v12) fragments were amplified from *N. caninum* cDNA and cloned within the pGEM-T-Easy vector (Promega). Primer sequences for cloning are summarized in

Supplementary table 2. All primers were purchased from Sigma-Aldrich, and the Expand High Fidelity Plus PCR System (Roche) was used for all PCRs. Amplicons were purified with the GENECLEAN Turbo kit (MP Biomedicals) from 1% low melting agarose gels (Lonza).

2.4. Production of recombinant proteins, mass spectrometry analysis and SDS-PAGE

E. coli NovaBlue Single Competent Cells (Novagen) were transformed with construct-containing plasmids, which were isolated using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced with an ABI Prism 377 DNA sequencer (Applied Biosystems) using T7 forward and reverse primers in the Genomics Unit of the Scientific Park of Madrid. All sequences were aligned with 100% consensus.

E. coli BL21(DE3) pLysS competent cells (Agilent Technologies) were transformed with the resulting expression vectors and foreign expression of rNcROP40 and rNcROP2Fam-1 as a (His)6-tagged fusion proteins was carried out following standard procedures (Álvarez-García *et al.*, 2007). Denatured proteins were on-column refolded and purified using HisTrapHP columns coupled to the ÄKTAprime Plus system (GE Healthcare) as previously described (Pastor-Fernández *et al.*, 2015a). Recombinant proteins included the whole NcROP40 sequence (1-392 aa) and the C-terminus domains for rNcROP2Fam-1 (238-594 aa), excluding the RAH domains. Concentration and purity of recombinant proteins was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a standard BSA scale (Roche) and using the GS-800 densitometer coupled to the Quantity One software (Bio-Rad Laboratories) (Álvarez-García *et al.*, 2007). Electrophoresed proteins were manually excised from prepared Coomassie-stained 1-D gels for mass spectrometry (MS) analysis (peptide mass fingerprinting) following standard procedures (Risco-Castillo *et al.*, 2007).

2.5. Polyclonal antibody production and affinity purification

Polyclonal sera against rNcROP40 (Regidor-Cerrillo *et al.*, 2012) and rNcROP2Fam-1 were raised in New Zealand White rabbits (Harlan Laboratories) following a procedure previously described (Risco-Castillo *et al.*, 2007). Samples of pre-immune serum were collected to confirm the absence of antibodies against *N. caninum* by Western-Blot. All protocols followed the proceedings detailed by the current legislation at the time of the experiment (Spanish Royal Decree 1201/2005) and were approved by the Animal Research Committee of the Complutense University. Affinity purified antibodies were prepared from polyclonal antibodies (PAbs) following standard procedures (Álvarez-García *et al.*, 2007).

2.6. Immunoblots

Detection of NcROP40 and NcROP2Fam-1 proteins in parasite extracts was carried out by Western-Blot following SDS-PAGE under reducing conditions. Unless otherwise stated, all reagents were purchased from Bio-Rad Laboratories. 2×10^7 purified Nc-Liv tachyzoites were disrupted by bath-sonication, electrophoresed in 15% bis-acrylamide gels and transferred onto nitrocellulose membranes according to standard procedures. PAbs α-rNcROP40 and α-rNcROP2Fam-1 were diluted

at 1:5,000. Goat anti-rabbit IgG antibody conjugated to peroxidase (Sigma-Aldrich) was used as secondary antibody at 1:25,000 dilution. Reactions were developed by chemiluminiscence with the Immobilon Western Chemiluminescent HRP Substrate (Millipore). For image acquisition, AGFA films (Curix/RP2 Plus) and AGFA CP1000 processor were used after 1 to 30 seconds of exposure time.

2.7. Immunogold-labeling and transmission electron microscopy (TEM)

Infected keratinocyte cultures were fixed and LR-White embedded and on-section labeled as previously described (Risco-Castillo *et al.*, 2007). Affinity-purified rabbit α-rNcROP40 was diluted 1:2 in PBS-0.3% BSA and sections were incubated for 1 h in a moist chamber. They were then washed in three changes of PBS, 10 min each, and goat anti-rabbit conjugated to 10 nm diameter gold particles (Amersham) was applied at a dilution of 1:5 in PBS-0.3% BSA as secondary antibody. After another 3 washes, 10 min each, grids were air dried and contrasted with uranyle acetate and lead citrate (Hemphill *et al.*, 2004). Specimens were viewed on a Phillips 600 TEM operating at 60 kV.

2.8. Immunofluorescence staining

Protein localization dynamics in *N. caninum* tachyzoites were studied on infected MARC-145 cells on rounded coverslips at different time-points after infection. A total of 5×10^4 cells were placed on sterile 13 mm-coverslips onto 24-well plates and incubated overnight at 37ºC on a 5% CO_2 atmosphere. Then, tachyzoites were scrapped from culture flasks, passed through a 21-gauge needle and counted on a haemacytometer by trypan blue exclusion. Subsequently, MARC-145 monolayers were infected with *N. caninum* for 20 and 40 min (MOI 3), 1, 2, 6 (MOI 3), 24, 32 (MOI 2) and 48 h (MOI 1). After infection, non-adherent parasites were removed from coverslips by three PBS washes. Then, three methods of fixation were employed. Absolute methanol, 2% paraformaldehyde in PBS and 2% paraformaldehyde-0.05% glutaraldehyde in PBS were used as fixatives for 10 to 30 minutes at room temperature. All samples were immediately processed for immune-fluorescence staining.

Coverslips were blocked and permeabilised with PBS containing 3% bovine serum albumin (Roche) and 0.25% Triton X-100 (Merck Chemicals) for 30 min at 37ºC. Then, cultures were labelled with the monoclonal antibody (MAb) α-NcSAG1 as a surface marker (1:250 dilution) (Björkman & Hemphill, 1998) and affinity purified PAbs α-NcROP40 and α-NcROP2Fam-1 (1:8 dilution) by incubation for 1 h at room temperature. Following three washes with PBS, coverslips were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG at 1:1,000 dilution (Molecular Probes) for 1 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole dye (DAPI, Lonza) at 1:5,000 dilution in PBS. Finally, coverslips were mounted on glass slides with ProLong® Gold antifade reagent (Molecular Probes). Evacuoles were detected on infected HFFs in the presence of cythochalasin D following the same protocol (Supplementary Figure 3).

To phalloidin staining, coverslips fixed in 2% paraformaldehyde were blocked, permeabilised

and labelled with MAb α-NcSAG1 and affinity purified PAb α-NcROP2Fam-1 as described above. After washing, they were incubated with Alexa Fluor 647-conjugated goat anti-mouse IgG (1:1,000), Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000), phalloidin–TRITC (1:250) (Sigma-Aldrich) and DAPI dyed.

Single 1µm slices of immunofluorescence stainings were captured with a Leica TCS-SPE confocal laser-scanning microscope (Leica Microsystems) in the Department of Biochemistry and Molecular Biology IV of the Complutense University (Madrid). Image processing was performed using the LAS AF (Leica Microsystems) and the ImageJ software (NCBI, http://rsb.info.nih.gov/ij/).

2.9. Secretion assays

Secretion assays were performed with tachyzoites obtained from cultures prior to egress. For this purpose, parasites were scrapped from culture flasks, pelleted by centrifugation (1350 × g, 10 min, 4º C), passed through a 21-gauge needle and purified by PD-10 desalting columns (GE Healthcare). Then, 1×10^8 tachyzoites were placed on 500 µl of cold phenol red-free DMEM (Life-Technologies) and stimulated with either 10 µM A23187 (Sigma-Aldrich), 1% ethanol (Merck Chemicals), or 10 mM dithiothreitol (DTT, Calbiochem) for 20 minutes at 37º C (Naguleswaran *et al.*, 2001). Non-stimulated parasites were kept on ice during the same period of time. After the incubation, secretion supernatants were recovered by double centrifugation (1350 × g, 10 min, 4º C and 8000 × g, 10 min, 4º C), passed through 0.2 µm PVDF filters (Whatman, GE Healthcare) and supplemented with phosphatase and protease inhibitor cocktails (Sigma-Aldrich). Pelleted parasites were washed once in cold PBS supplemented with phosphatase and protease inhibitor cocktails, and recovered by centrifugation (1350 × g, 10 min, 4º C). All samples were stored at -80º C until further analyses.

Supernatants and pellets were analysed by immunoblotting, and secretion was estimated by comparing equal amounts of secretion supernatants and tachyzoite lysates. Monoclonal antibodies directed against NcTUBα (α-TUBα MAb, Sigma-Aldrich) were used on immunoblots of secreted supernatant fractions to monitor tachyzoite lysis, and antibodies directed against NcMIC2 were used as a positive control of secretion (Lovett *et al.*, 2000). The α-TUBα MAb specifically recognized NcTUBα protein in tachyzoite extracts. PVDF membranes were incubated with rabbit α-rNcROP40, α-rNcROP2Fam-1 and α-rNcMIC2 at 1:5,000 dilutions, whereas α-TUBα MAb was employed at 1:10,000 dilution. Secondary antibodies were employed at 1:25,000 (goat anti-rabbit IgG antibody conjugated to peroxidase) and at 1:80,000 dilutions (goat anti-mouse IgG antibody conjugated to peroxidase) (Sigma-Aldrich). Reactions were developed by chemiluminiscence with the Immobilon Western Chemiluminescent HRP Substrate as describe above.

*2.10. Evaluation of Nc*ROP40 *and Nc*ROP2 Fam-1 *mRNA expression levels*

The mRNA expression levels of Nc*ROP40* and Nc*ROP2Fam-1* were assessed by real-time reverse transcription PCR throughout the lytic cycle of tachyzoites at four representative points

which illustrate the recent invasion, PV formation and maturation, exponential growth of parasites and tachyzoite egress. For this purpose, MARC-145 cultures were infected with the Nc-Liv isolate at MOI 3 for 6, 24, 48 and 56 h. Infected cultures were synchronised by washing the monolayer twice with pre-warmed PBS and replacing the culture media at 6 hours post-infection (hpi), to remove non-adherent parasites. Cells were harvested with a cell scraper and recovered by centrifugation at 1,350 × *g* for 15 minutes at 4º C. Pelleted parasites were conserved at -80º C until RNA extraction. The experiment was carried out in triplicate. For each experiment, three different flasks were analysed at each time-point. The effect of induced egress of tachyzoites on expression levels of Nc*ROP40* and *NcROP2Fam-1* was also studied in parallel. For this purpose, five flasks from three different experiments containing cells that were infected for 48 h were treated with 10 mM DTT for 1 h, after which tachyzoites had undergone egress from approximately 80% of parasitophorous vacuoles. Tachyzoites were then recovered as described above.

Total RNA was extracted using the Maxwell® 16 LEV simplyRNA Purification Kit (Promega), that includes a DNAse treatment, following the manufacturer's recommendations. RNA concentrations were determined by spectrophotometry (Nanophotometer, Implen), and RNA integrity was checked by the visualization of the 18S and 28S ribosomal fragments after electrophoresis on 1% agarose gels. Reverse transcription was carried out by the master mix SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) in a 20 µl reaction using 2.5 µg of total RNA. Resulting cDNA was diluted 1:20 and analysed by real-time PCR.

Real-time PCR reactions were performed using the Power SYBR® Green PCR Master Mix in the ABI 7300 Real Time PCR System (Applied Biosystems) following standard conditions. Primers used for amplification of Nc*ROP40*, Nc*ROP2Fam-1* and the housekeeping genes Nc*TUBα* and Nc*SAG1* are shown in Table 1. A seven-point duplicate standard curve based on 10-fold serial dilutions was included on each run. pET45b(+)-Nc*ROP40*, pET45b(+)-Nc*ROP2Fam-1*, pGEM-T-Nc*TUBα* and pGEM-T-Nc*SAG1* plasmids were used as standards.

mRNA expression levels for each target were normalized by the -ΔCt method (Schmittgen & Livak, 2008). -ΔCt values were calculated by subtracting the Ct value of the normalizer genes from the Ct value of each sample. Relative fold increases or decreases were assessed by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Since expression levels at 24 hpi were the lowest for both proteins, the -ΔΔCt value was calculated by subtracting the mean -ΔCt values for each protein at 24 hpi as baseline samples as indicated in this formula: -ΔΔCt = -[(Ct Nc*ROPx* - Ct Nc*TUBα*) - (mean Ct Nc*ROPx* at 24 h - mean Ct Nc*TUBα* at 24 h)]. Raw RNA samples were included in each batch of amplifications to confirm the absence of *N. caninum* genomic DNA. Data analyses of mRNA expression levels were carried out by Kruskal-Wallis and Dunn's tests using GraphPad Prism v6.01 software.

Table 1: Primers used to amplify Nc*ROP40*, Nc*ROP2Fam-1*, Nc*SAG1* and Nc*Tubilin alpha* sequences by real time-PCR.

Protein	ToxoDB accession no.	Primer sequences	Reference	Length	Introns [a]	Slope [b]	R^2 [b]
Nc*ROP40*	NCLIV_012920	CATCAAGCAGCCCAGAATCA TGGTGACTGCGACCAACTTA	This study	94 bp. 1021-1114	No	-3.71	0.996
Nc*ROP2Fam-1*	NCLIV_001970	TTCTTCCTCTCCAAGCGACA TTGAGTCGTTCCCGAAGTTG	(Alaeddine *et al.*, 2013)	140 bp. 1604-1743	No	-3.68	0.996
Nc*SAG1*	NCLIV_033230	CGGTGTCGCAATGTGCTCTT ACGGTCGTCCCAGAACAAAC	(Fernández-García *et al.*, 2006)	150 bp. 504-653	No	-3.24	0.997
Nc*TUBα*	NCLIV_058890	GGTAACGCCTGCTGGGAG GCTCCAAATCCAAGAAGACGCA	(Alaeddine *et al.*, 2013)	166 bp. 49-214	Yes*	-3.24	0.994

[a] Primers for intron-containing sequences were designed using cDNA as template. * Forward primer for Nc*TUBα* amplification annealed at intron splice junction to prevent amplification of genomic DNA.

[b] Descriptive values of real time-PCR from standard curves for each pair of primers are shown.

2.11. Phosphorylation assays

Phosphorylation assays were performed on denatured lysates generated from infected MARC-145 cell cultures at 56 hpi, when tachyzoite had escaped from parasitophorous vacuoles and invaded neighboring cells. Freshly pelleted cell monolayers were resuspended on alkaline phosphatase-compatible buffer (100 mM sodium chloride [Panreac], 50 mM Tris-HCl [Panreac], 10 mM magnesium chloride [Merck Chemicals], 1 mM DTT [Calbiochem], 0.2 % Triton X-100 [Merck Chemicals] and protease inhibitor cocktail [Sigma-Aldrich], pH 7.9) or in phosphatase inhibitor buffer (50 mM HEPES [Sigma-Aldrich], 100 mM sodium fluoride [Sigma-Aldrich], 2 mM sodium orthovanadate [Sigma-Aldrich], 2 mM EDTA [Sigma-Aldrich], 1 mM DTT, 0.2 % Triton X-100 and protease inhibitor cocktail). Extracts were disrupted on ice for 15 minutes by bath-sonication (Ultrasons, Selecta) and shaked by vortexing during an additional 45 minutes. Alkaline phosphatase treatment (20 U CIP/2×10^7 tachyzoites, New England Biolabs) was only applied on extracts resuspended in alkaline phosphatase-compatible buffer for 90 minutes at 37º C. Resulting extracts were stored at -80ºC until further analysis.

Phosphorylated proteins experience a mobility shift on Phos-tag SDS-PAGE electrophoresis (Kinoshita *et al.*, 2006). To determine if NcROP40 and NcROP2Fam-1 are phosphorylated, tachyzoite extracts resuspended in alkaline phosphatase-compatible buffer (CIP) and phosphatase inhibitor buffer (PI) were electrophoresed in 15% bis-acrylamide gels supplemented with 25 µM Phos-Tag (Wako Pure Chemicals Industries) and 50 µM manganese (II) chloride (Merck Chemicals). After electrophoresis, gels were washed once in 0.1 M EDTA in transfer buffer and once in transfer buffer without EDTA to remove metal complexes. Then, gels were transferred onto nitrocellulose membranes according to standard procedures. Membranes were incubated with α-rNcROP40 and

α-rNcROP2Fam-1 at 1:1,000 dilution, and then incubated with goat anti-rabbit IgG antibody conjugated to peroxidase at 1:1,000 dilution. Reactions were developed using 4-chloro-1-naphtol as substrate until signal visualization.

3. Results

3.1. In silico *analysis and Nc*ROP40 *sequencing*

NcROP40 (NCLIV_012920, chromosome V) is currently classified as an unspecified product, but in previous releases of ToxoDB (v7.3) the protein was named as rhoptry kinase family protein ROP40 and considered as orthologous gene of TgROP40. No introns are predicted in the Nc*ROP40* sequence, which contains 1176 bp and codes for a product of a predicted molecular weight of 43 kDa. In contrast, the Tg*ROP40* sequence (TGME49_291960, chromosome IX) contains two introns, a coding sequence of 1578 bp and a predicted molecular weight of 57.9 kDa. However, according to previous releases of ToxoDB (v7.3), the Tg*ROP40* gene (TGME49_091960) has no introns. The predicted peptide sequences of NcROP40 and TgROP40 proteins (v7.3) share 32.3% identity and 48.7% similarity. In addition, a Pfam database search identified a protein kinase-like domain in NcROP40, but catalytic activity is lacking (PANDIT: PF14531).

Due to the observed inconsistencies between Nc*ROP40* and Tg*ROP40*, the chromosome V sequence of the *N. caninum* Nc-Liv genome was analyzed in detail. First, the N-terminus of the Nc*ROP40* sequence was strikingly shorter than that annotated for Tg*ROP40* (~400 bp). In order to elucidate these differences, up and down-stream Nc*ROP40* sequences (from positions 662772 to 665947, chromosome V) were submitted to the ORF Finder tool, which displayed a unique 1578 bp ORF, which corresponds to a putative protein with a calculated molecular weight of 57.8 kDa. This finding is consistent with the Tg*ROP40* sequence. The presence of the additional N-terminal fragment in the Nc*ROP40* ORF was confirmed by RT-PCR using cDNA from tachyzoites at two different time points of infection (Supplementary Figure 1). The newly identified ORF (now termed Nc*ROP40-long*) contains the Nc*ROP40* sequence as listed in ToxoDB and an additional 402 bp at its N-terminus (Supplementary Figure 2) (GenBank: KP731805, KP731806 and KP731807). According to the previous Tg*ROP40* gene (TGME49_091960 in ToxoDB v7.3), no introns were predicted when the Nc*ROP40-long* DNA sequence was submitted to the Splign tool. The percentage of amino acid sequence identity between TgROP40 and NcROP40-long increased from 32.3 to 42.9, whilst similarity increased from 46.3% to 61.4% (Figure 1A).

Protein trans-membrane regions were predicted for the NcROP40-long protein between the positions 5 and 25, but according to SignalP predictions the signal peptide is cleaved between amino acids (aa) 19 and 20. Sequence comparison among the most representative members of the ROP2-family (Figure 1B), as well as alpha helices prediction within the structure of the NcROP40-long protein (Figure 1C) suggest that the protein lacks RAH domains. Phosphorylation sites both in NcROP40-long and NcROP2Fam-1 were subjected to three different prediction programs (NetPhos v2.0, NetPhosK

v1.0, and Diphos v1.3.), and were only considered when detected by at least two of them. In this sense, NcROP40 showed two putative phosphorylation sites at position S-75 and S-78, whilst phosphorylation of NcROP2Fam-1 was predicted to occur at positions S-82 and S-129.

The Nc*ROP40-long* ORF and its up and down-stream sequences were amplified by PCR from DNA of three different isolates, which have shown manifest differences in virulence: Nc-Liv, Nc-Spain7 and Nc-Spain1H. Primers were designed to amplify a fragment of 2918 bp, containing the ORF. After PCR, amplicons were sequenced and analyzed in detail. For all isolates a 2362 bp consensus fragment was sequenced in two directions. This fragment comprised the Nc*ROP40-long* ORF (1578 bp) and an additional 148 and 636 bp in its flanking regions. Thus, comparative analyses did not show differences in the amplified sequences among the three isolates (Supplementary Figure 2).

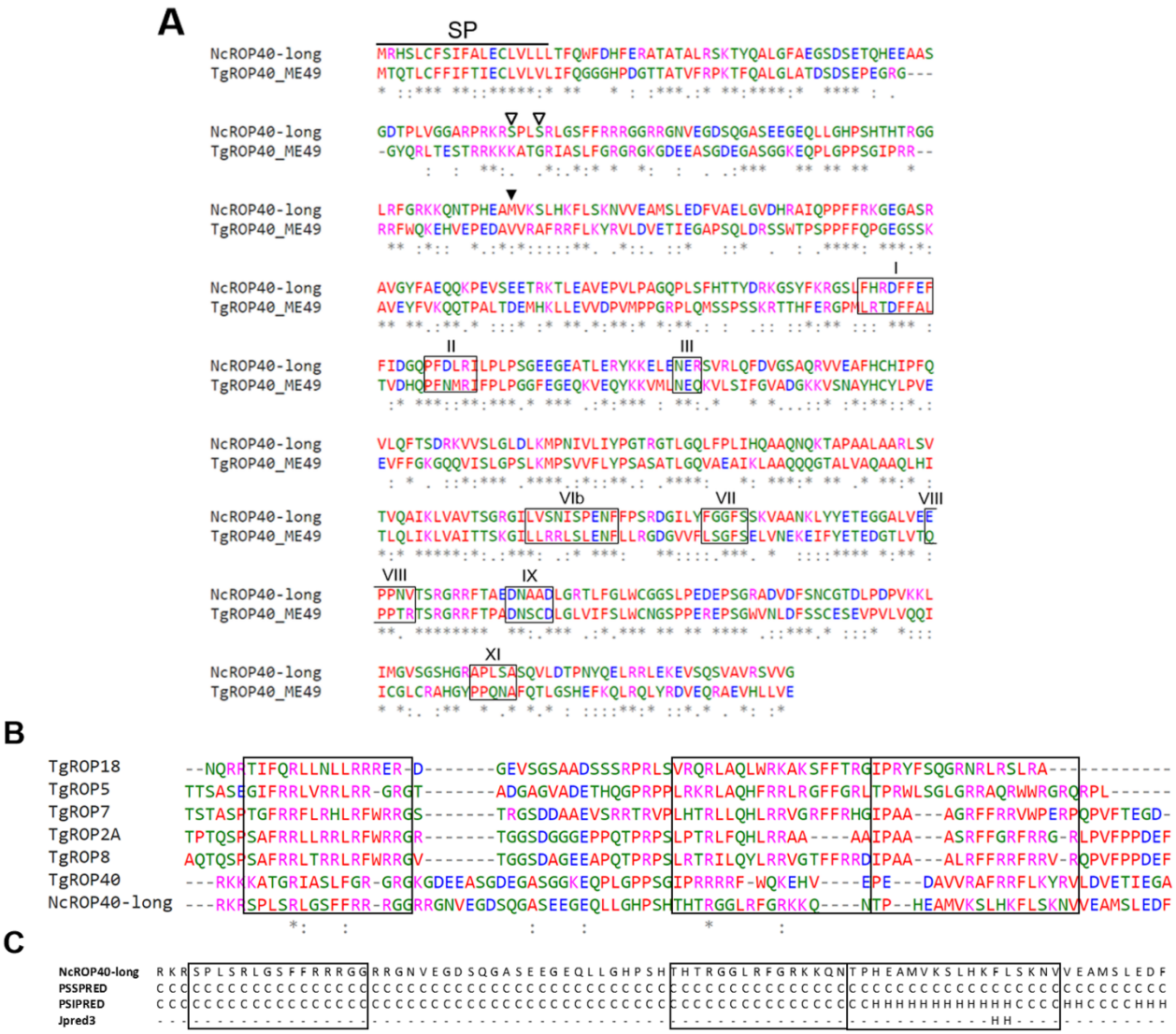

Figure 1. **A:** Sequence alignment of the ROP40 protein, both in *N. caninum* (NCLIV_012920) and *T. gondii* (TGME49_091960). NcROP40-long is referred to the NcROP40 sequence incorporating and additional 134 aminoacids in the N-terminus. SP: signal peptide. Empty arrow head: potential phosphorylation sites. Filled arrow head: origin of the NcROP40 protein as shown in ToxoDB. Boxes and roman numerals: conserved motifs of likely inactive rhoptry kinase regions as described for Talevich and colleagues in 2013. **B:** Comparison of the RAH domains among rhoptry proteins from the ROP2-family. Boxes designate the three domains described for El Hajj and colleagues in 2006. Sequences were obtained from ToxoDB with the following accession numbers: TGME49_005250 (TgROP18), TGME49_108080 (TgROP5), TGME49_095110 (TgROP7), TGME49_015780 (TgROP2A), TGME49_015770 (TgROP8), TGME49_091960 (TgROP40) and NCLIV_012920 (NcROP40). For A and B asterisks (*) indicate fully conserved residues, whilst colons (:) and periods (·) indicate conservation between groups of strongly or weakly similar properties, respectively. **C:** Secondary structure predictions of the NcROP40-long RAH domains by PSSpred, PSIPRED and Jpred3 servers. H: helix. C: coil. Dashes: undefined.

3.2. Protein sequence and immunodetection

The identities of rNcROP40 and rNcROP2Fam-1 were confirmed by mass spectrometric analyses. rNcROP40 matched with the NCLIV_012920 sequence (score: 175; 18/60 matched values; 64% of sequence coverage), whereas rNcROP2Fam-1 matched with the NCLIV_001970 annotation (score: 345; 33/65 matched values; 69% of sequence coverage). These analyses corroborated the composition of both proteins, with a molecular weight of 43.9 and 43.2 kDa respectively, according to the predicted molecular weights of rNcROP40 and rNcROP2Fam-1, which exclude part of their N-terminal domains. Hence, rNcROP40 and rNcROP2Fam-1 were used to develop PAbs in rabbits. *N. caninum* tachyzoite crude extracts were separated by SDS-PAGE under reducing conditions. Western blots revealed that α-NcROP40 reacted with five distinct bands of approximately 53, 44, 38, 32 and 28 kDa. The polyclonal α-NcROP2Fam-1 antiserum detected six different bands of approximately 58, 48, 40, 36, 34 and 26 kDa (Figure 2).

3.3. Subcellular localization of NcROP40 by TEM

In order to confirm the subcellular localization of NcROP40, immunogold-TEM was carried out on sections of keratinocytes infected with *N. caninum* tachyzoites, and of cultures infected with bradyzoites generated *in vitro* by sodium nitroprusside treatment. In both, *N. caninum* tachyzoites (Figure 3, A-B) and *in vitro* induced-bradyzoites (Figure 3, C-D), affinity-purified anti-NcROP40 antibodies localized to rhoptry bulbs.

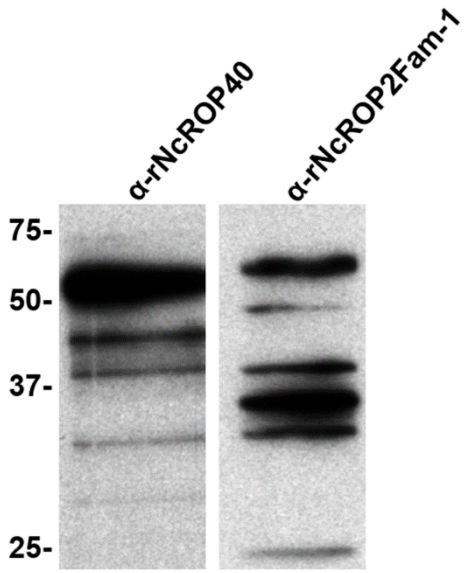

Figure 2: *N. caninum*-based Western-blot showing the immuno-reactivity of α-rNcROP40 and α-rNcROP2Fam-1 antibodies. Five bands of approximately 53, 44, 38, 32 and 28 kDa were detected with α-NcROP40 antibodies, whilst six different bands of approximately 58, 48, 40, 36, 34 and 26 kDa were detected with α-NcROP2Fam-1 antibodies.

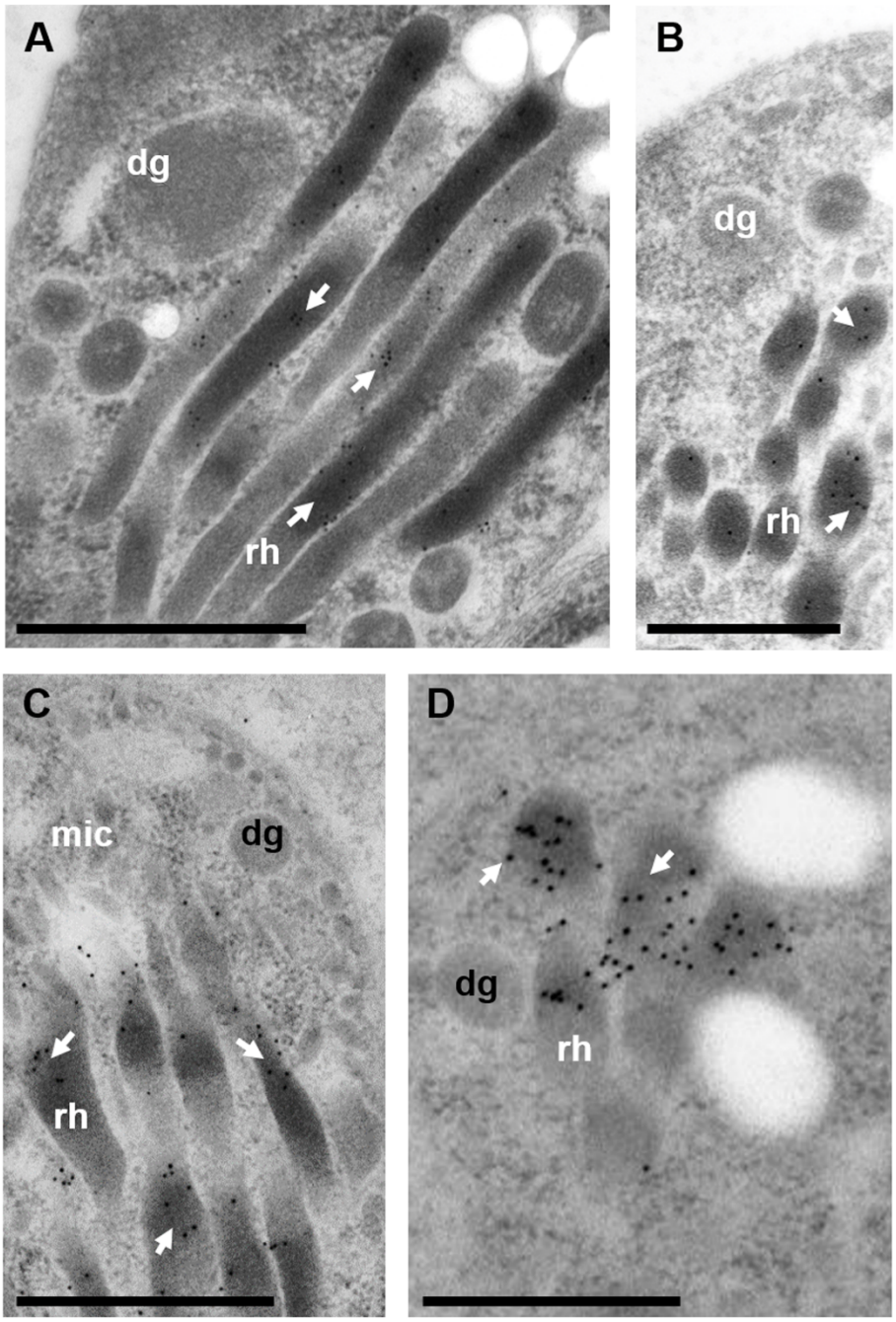

Figure 3. NcROP40 is a rhoptry protein associated with rhoptry bulbs. Transmission electron microscopy and immunogold staining in tachyzoites (A-B) and bradyzoites (C-D). Rhoptries (rh), dense granules (dg) and micronemes (mic) are indicated on the pictures. Bars represent 1 µm.

3.4. NcROP40 and NcROP2Fam-1 tracing throughout the lytic cycle

Immunofluorescence staining of NcROP40 on *N. caninum*-infected cultures showed a rhoptry-like pattern in tachyzoites throughout the lytic cycle, from 20 minutes to 56 hpi. Methanol fixation showed the clearest results in terms of NcROP40 immunolocalization. In contrast, fixation with paraformaldehyde and glutaraldehyde mixtures resulted in a lower

staining intensity (Figure 4). The rhoptry-like pattern was clearly associated with the apical end of tachyzoites in all the micrographs, and disappeared as the captured slices intersected the parasites in more external areas (Figure 4, 24 and 32 hpi, MeOH fixation). Interestingly, the presence of NcROP40 was not detected, neither in evacuoles during the invasion phases nor in the PVM during the development and establishment of the PV, with similar results obtained using three different fixation protocols. Hence, no secretion of NcROP40 protein could be detected under the tested conditions.

Concerning NcROP2Fam-1, our PAbs specifically recognized rhoptry-like structures at the parasite apex at all time-points as described for NcROP40. In contrast, secretion of the protein was detected from 20 min to 24 hpi using all three fixation protocols (Figure 5). Specifically, evacuoles were detected from 20 minutes to 6 hpi. These rhoptry-derived secretory vesicles were localized intracellularly and surrounded the host cell nucleus, as shown by the phalloidin stainings in those coverslips fixed with paraformaldehyde (Figure 5, 6 h, PFA fixation). At later time points, during the establishment of the PV, NcROP2Fam-1 was detected on the PVM (Figure 5, 6 hpi) and in the PV matrix (Figure 5, 24 hpi) under all the fixation methods. Thereafter, NcROP2Fam-1 was restricted to rhoptries (Figure 5, 32 and 48 hpi). Interestingly, the protein was released again during egress, where it appeared to localize on the surface of the extracellular tachyzoites (Figure 5, 56 hpi).

Identical results were obtained for NcROP40 and NcROP2Fam-1 proteins by specific evacuole assays carried out in human foreskin fibroblasts (Supplementary Figure 3).

3.5. Induced secretion of NcROP40 and NcROP2Fam-1

Freshly purified tachyzoites were treated with A23187, ethanol or DTT in order to induce the calcium-related protein secretion from apical organelles. However, NcROP40 and NcROP2Fam-1 proteins were not detected on secretome supernatants by immunoblotting. In contrast, a manifest secretion of the NcMIC2 protein was observed under the same conditions, according to previous findings (Lovett *et al.*, 2000). Moreover, inadvertent lysis of tachyzoites during these secretion assays could be discarded since no NcTUBα could be detected in any of the secreted fractions (Supplementary Figure 4).

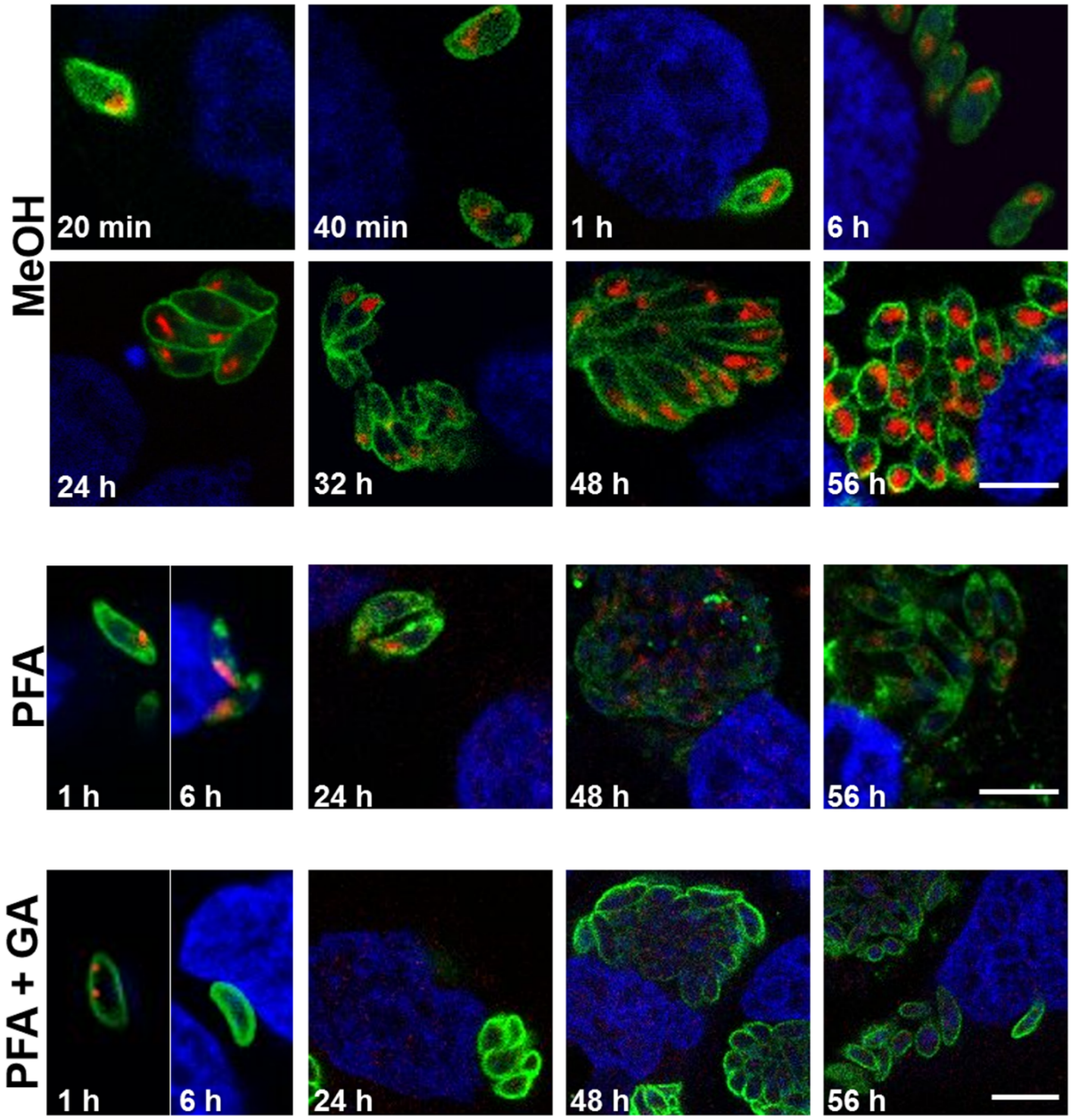

Figure 4. Confocal laser scanning microscopy of NcROP40 along the lytic cycle of tachyzoites. Infected cultures were fixed with metanol (MeOH), paraformaldehyde (PFA) and paraformaldehyde combined with glutaraldehyde (PFA+GA) and double labelled with affinity purified antibodies against NcROP40 (red) and monoclonal antibodies against NcSAG1 (green). Nuclei were stained with DAPI (blue). All the images show a single 1 µm slice. Bars represent 4 µm.

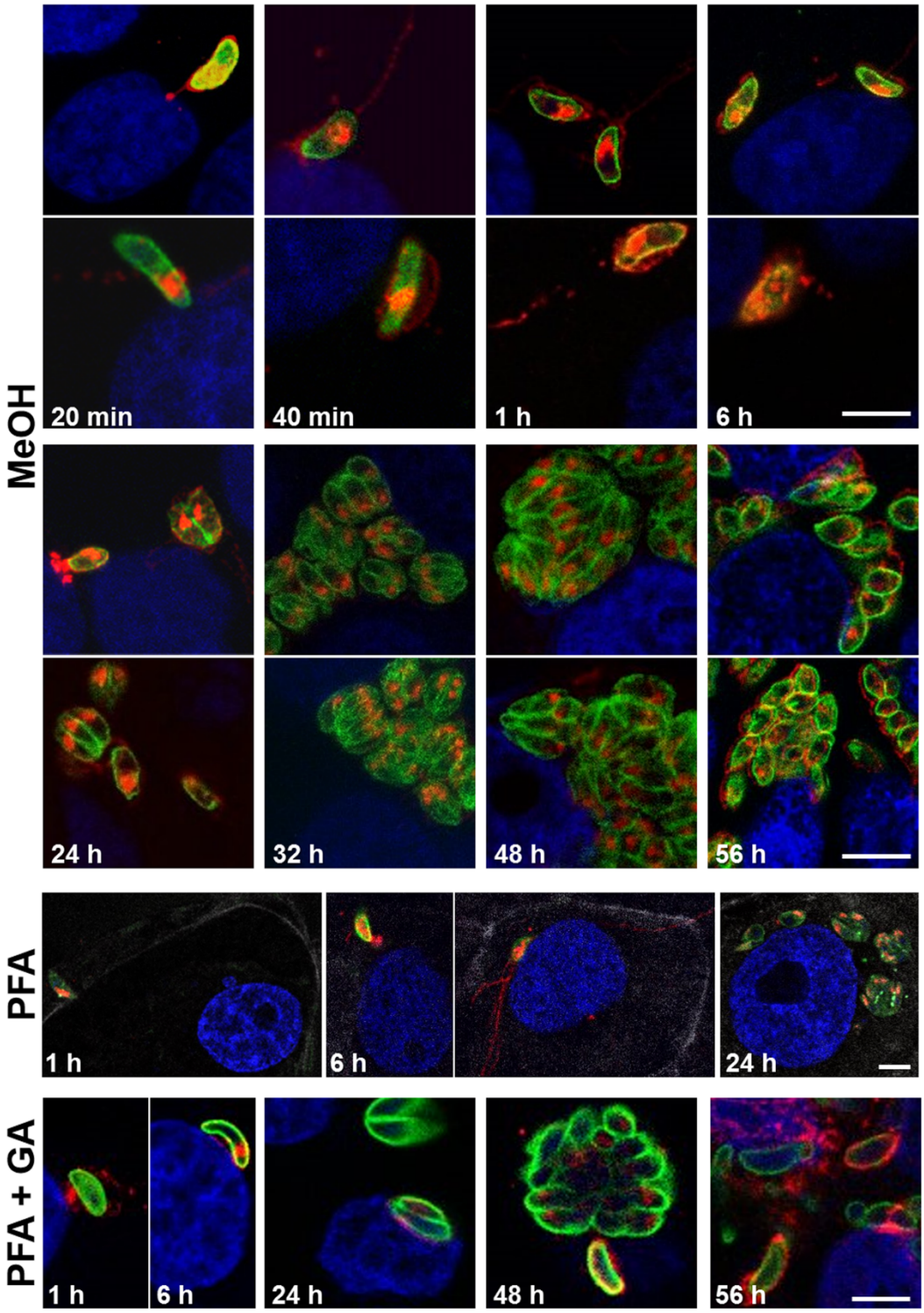

Figure 5. Confocal laser scanning microscopy of NcROP2Fam-1 along the lytic cycle of tachyzoites. Infected cultures were fixed with metanol (MeOH), paraformaldehyde (PFA) and paraformaldehyde combined with glutaraldehyde (PFA+GA) and double labelled with affinity purified antibodies against NcROP2Fam-1 (red) and monoclonal antibodies against NcSAG1 (green). Nuclei were stained with DAPI (blue). PFA-fixed cultures were also labelled with phalloidin to delimitate host-cell surface (white). All the images show a single 1 µm slice. Bars represent 4 µm.

*3.6. Nc*ROP40 *and Nc*ROP2Fam-1 *mRNA expression during the lytic cycle*

Nc*ROP40* and Nc*ROP2Fam-1* transcription levels were monitored at four representative time points during the lytic cycle. Similar results in the mRNA pattern were observed using Nc*TUBα* and Nc*SAG1* as normalizer genes (data not shown). The results presented here were processed using Nc*TUBα* as normalizer.

The lowest Nc*ROP40* and Nc*ROP2Fam-1* mRNA levels were found at 24 hpi. In contrast, mRNA levels were the highest at 6 hpi (during the invasion phase) and at 56 hpi (egress phase) for both proteins (P<0.005; Kruskal-Wallis test) (Figure 6, A). Differences in fold increases of mRNA transcription were calculated by the $2^{-\Delta\Delta Ct}$ method. Since the lowest normalized values for both Nc*ROP40* and Nc*ROP2Fam-1* were observed at 24 hpi, this time point was used as baseline to calculate the mRNA transcription fold increases during egress and invasion. Nc*ROP40* showed a 4-fold increase in mRNA levels at 6 and 56 hpi, and Nc*ROP2Fam-1* exhibited a 3-fold increase at the same time points. At 48 hpi, mRNA levels displayed a 2-fold increase for both Nc*ROP40* and Nc*ROP2Fam-1*.

Egress of *N. caninum* and *T. gondii* tachyzoites can be artificially induced *in vitro* by the addition of DTT into the culture medium. Thus, the effect of DTT supplementation on the expression of Nc*ROP40* and Nc*ROP2Fam-1* mRNA was studied. Different responses were observed: while Nc*ROP40* did not exhibit significant increases in its mRNA levels upon DTT treatment, Nc*ROP2Fam-1* mRNA transcription was significantly increased (P<0.005; Kruskal-Wallis test) (Figure 6, B). Moreover, mean values for Nc*ROP2Fam-1* mRNA remained above those observed at 56 hpi, while the corresponding values for NcROP40 remained below (Figure 6, B).

3.7. Phosphorylation of NcROP40 and NcROP2Fam-1 at the egress

The phosphorylation status of NcROP40 and NcROP2Fam-1 was studied at 56 hpi, as this was the time point when the mRNA levels for both proteins within the lytic cycle were the highest, simultaneously to tachyzoite egress and early invasion. Tachyzoites were harvested, processed under conditions that preserve the phosphorylation status, and extracts were separated by Phos-Tag SDS-PAGE electrophoresis. The electrophoretic mobility of NcROP40 on Phos-Tag gels was similar in both, alkaline phosphatase-treated and phosphatase inhibitor-treated extracts. In contrast, NcROP2Fam-1 showed a mobility shift in those extracts treated with phosphatase inhibitors, which suggests that NcROP2Fam-1 is phosphorylated at 56 hpi (Figure 7).

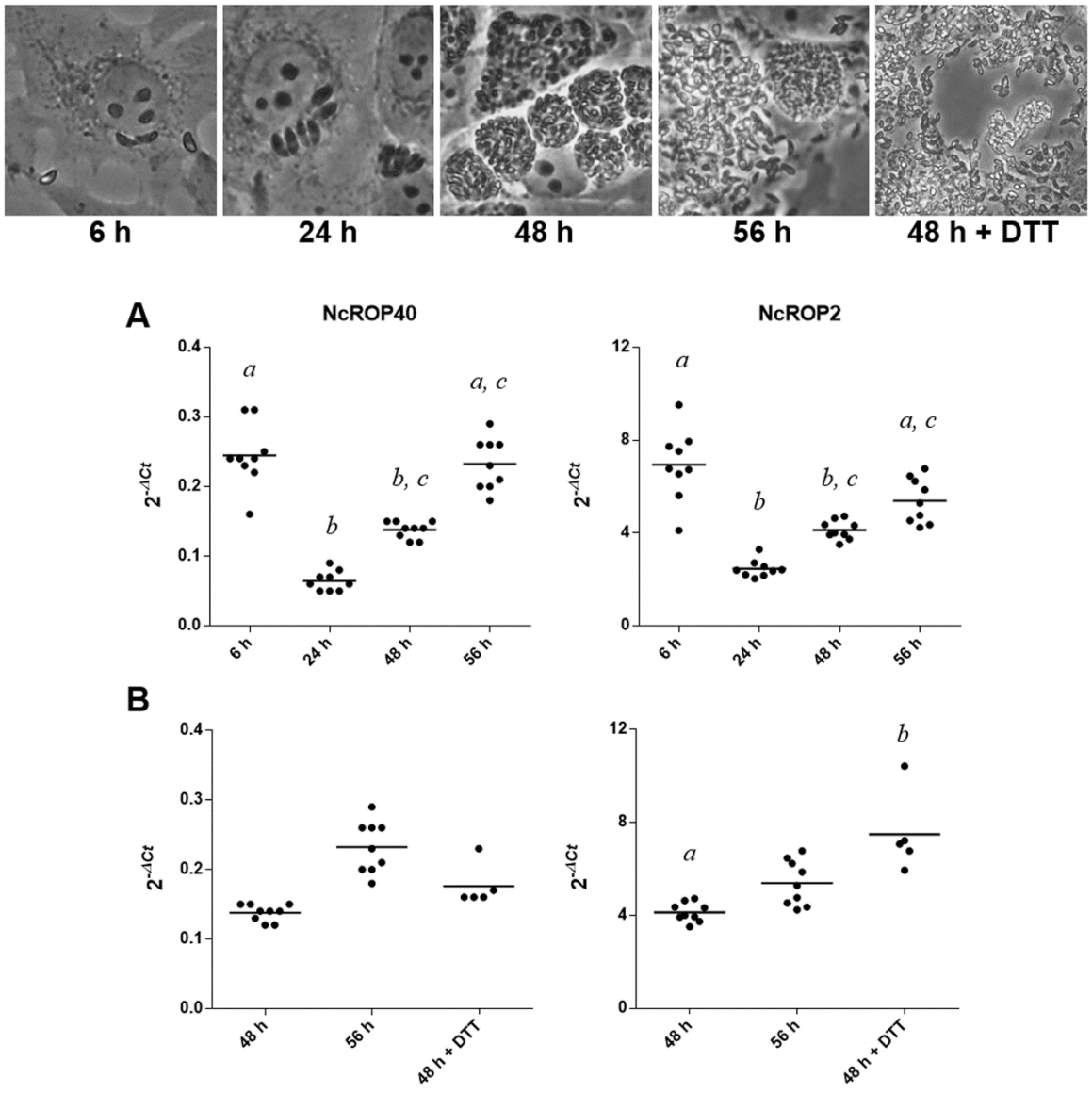

Figure 6. mRNA expression of Nc*ROP40* and Nc*ROP2Fam-1*. Real time-PCR was employed to assess the mRNA expression of both proteins along the lytic cycle. Top panel: photomicrographs showing the infection dynamics of the Nc-Liv isolate on MARC-145 cultures at recent invasion (6 h), PV formation and maturation (24 h), exponential growth of parasites (48 h) and tachyzoite egress (56 h and 48 h + DTT). A: mRNA expression levels of Nc*ROP40* and Nc*ROP2Fam-1* during the lytic cycle. B: Effect of DTT supplementation to artificially induce egress at 48 h on mRNA expression for both proteins. For A and B, each point represents a single sample and bars represent the mean value. *a*, *b* and *c* indicate significant differences (P<0.005; Kruskal-Wallis test).

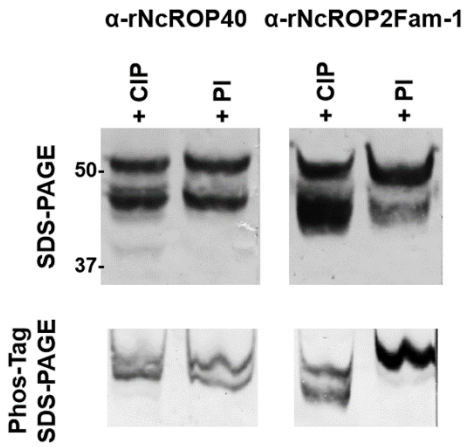

Figure 7: Phosphorylation detection of NcROP40 and NcROP2Fam-1 by Phos-Tag SDS-PAGE. Tachyzoite extracts obtained at 56 hpi were processed with alkaline phosphatase (CIP) and phosphatase inhibitors (PI), electrophoresed on SDS-PAGE and Phos-Tag SDS-PAGE gels, and blotted to nitrocellulose membranes. Then, both proteins were detected by their respective antibodies in order to detect a mobility shift of the proteins treated with PI.

4. Discussion

Considerable efforts have been undertaken to increase the understanding on how apicomplexan parasites interact with their host cells and how they maintain and optimize their intracellular life style. It is widely known that components of distinct secretory organelles, namely rhoptries, micronemes and dense granules, play a crucial role in defining the host-parasite relationship (Carruthers & Sibley, 1997) and therefore corresponding antigens are being extensively studied as vaccine targets to prevent infections by apicomplexan parasites.

TgROP proteins have shown to be important virulence factors (Lim *et al.*, 2012). In contrast, little is known about the rhoptry proteins in *N. caninum*. Several NcROP and NcRON proteins have been identified by different proteomic approaches and monoclonal antibodies (Regidor-Cerrillo *et al.*, 2012; Marugán-Hernández *et al.*, 2011a; Sohn *et al.*, 2011; Straub *et al.*, 2009), but only NcROP2Fam-1, which has been associated with the tachyzoites invasion process, has been partially characterized to date (Alaeddine *et al.*, 2013).

NcROP40 was shown to be more abundant in virulent isolates of *N. caninum* (Regidor-Cerrillo *et al.*, 2012). In *T. gondii*, limited information is available for the orthologous gene product Tg*ROP40* (initially named as TgROP2L6). Tg*ROP40* is highly expressed in tachyzoites (Peixoto *et al.*, 2010) and increased expression levels were observed during acute infections in mice (Pittman *et al.*, 2014). A number of studies suggested that NcROP40 is one of the major rhoptry components, since it has been detected by three different proteomic approaches (Pollo-Oliveira *et al.*, 2013; Regidor-Cerrillo *et al.*, 2012; Marugán-Hernández *et al.*, 2011a). Hence, NcROP40 expression could be an important element related to parasite virulence. Indeed, a vaccine formulation combining NcROP40+NcROP2Fam-1 recombinant proteins

was recently assessed in a pregnant mouse model of neosporosis and conferred partial protection against congenital transmission of *N. caninum*, with NcROP40 and NcROP2Fam-1 acting synergistically (Pastor-Fernández *et al.*, 2015a).

Comparison of the Nc*ROP40* ORF and its potential regulatory expression sequences among three different *N. caninum* isolates with differing virulence and *in vitro* behavior (Regidor-Cerrillo *et al.*, 2011; Pereira García-Melo *et al.*, 2010; Regidor-Cerrillo *et al.*, 2010a) did not reveal any polymorphism that could explain differences in virulence as described for TgROP18 (Steinfeldt *et al.*, 2010). However, dissimilarities in protein abundance among isolates might be due to regulatory mechanisms such as epigenetics, which have been shown to be involved in genome reprogramming during tachyzoite to bradyzoite conversion in *T. gondii* (Dixon *et al.*, 2010). These analyses allowed a detailed dissection of the Nc*ROP40* gene, including flanking regions, and lead to the description of the Nc*ROP40-long* sequence (Nc*ROP40* sequence with an additional 402 bp in its N-terminus), whose presence was confirmed by RT-PCR (Supplementary Figure 1). In addition, an improved transcriptome annotation for *NcROP40* has recently been submitted (GenBank: CEL65449.1), confirming our results (Ramaprasad *et al.*, 2015).

Inconsistencies in the measured (53 kDa) and theoretical (58 kDa) molecular weight of NcROP40-long, as well as the presence of different bands on immunoblots, could reflect the maturation process described for all ROP2-family rhoptry proteins, which are synthesized as pro-proteins (Hajagos *et al.*, 2012). In fact, Alaeddine and colleagues described the processing of the NcROP2Fam-1 protein by Western-blot through different affinity purified antibodies directed against peptides located at the C-terminal end of the protein (Alaeddine *et al.*, 2013). In *T. gondii* TgSUB2 protease is in charge to remove the N-terminal domains that are involved in rhoptry targeting at a highly conserved SΦx(E/D) site (Hajagos *et al.*, 2012). This sequence was also found in the N-terminal domain of NcROP2Fam-1 (Alaeddine *et al.*, 2013), but is absent in NcROP40. In any case, the polyclonal antibody recognizes a main band of 53 kDa and a number of additional bands by Western blotting, which may reflect a protein maturation process. Nevertheless, further studies must be carried out to define more accurately the implication of these changes on protein function.

In silico analyses are useful to predict certain protein features, and were employed in this study to further characterize the NcROP40 protein. The presence of a signal peptide is an important pre-requisite for a protein to enter the secretory pathway in eukaryotes, and putative signal peptides are present in both TgROP40 (El Hajj *et al.*, 2006) and NcROP40 (this work). This is in contrast with our observations, since NcROP40 secretion is not detected by immunofluorescence microscopy, even when employing three different fixation protocols or specific evacuole assays. Moreover, NcROP40 does not interact with the PVM, albeit this finding is consistent with the predicted lack of RAH domains in its sequence. RAH domains are the regions displaying the highest similarities between each of the members of the ROP2-family, and are required for PVM association.

These domains are also absent in the TgROP40 protein, and consequently it does not associate with the PVM (Reese & Boothroyd, 2009; El Hajj *et al.*, 2006). Nevertheless, previous studies carried out with the toxofilin protein of *T. gondii* showed that secretion of low abundance proteins may be undetectable by immunofluorescence approaches, which is especially relevant for those proteins that are not concentrated on a membrane or in an intracellular compartment (Lodoen *et al.*, 2010). This may be the case for the NcROP40 protein that could be secreted into the host cell cytosol. Interestingly, the TgROP40 protein shows some nuclear localization when is heterologously expressed in infected HFF, suggesting that the protein may be translocated into the host cell nucleus after its secretion in the cytosol. Nevertheless, the immunodetection of the NcROP40 into the host cell nucleus was not achieved in this study (Reese & Boothroyd, 2009). In contrast to NcROP40, NcROP2Fam-1 was extensively secreted under the tested conditions (Alaeddine *et al.*, 2013). The protein was easily detected in evacuoles, and then surrounding invasive tachyzoites from 1 to 24 hpi. Similar findings have been described for the TgROP2 protein, which may participate in the PVM formation (Dunn *et al.*, 2008; Beckers *et al.*, 1994). Consistent with our findings, most of the rhoptry proteins described to date in *T. gondii* are secreted and participate in host cell invasion, PV formation and maturation, and/or are involved in hijacking the host cell machinery (Kemp *et al.*, 2013).

On the other hand, we could not detect any NcROP40 and NcROP2Fam-1 protein in the secretory fractions after induction of tachyzoite secretion using A23187, ethanol or DTT stimulation. This indicates that rhoptry discharge is not affected by elevated intracellular calcium levels as previously stated for *T. gondii* rhoptry proteins (Carruthers & Sibley, 1999), and that rhoptry secretion can be only induced upon host cell contact.

The mRNA levels of Nc*ROP40* and Nc*ROP2Fam-1* transcripts were quantified during defined time points of the lytic cycle of tachyzoites grown in MARC-145 cells (Regidor-Cerrillo *et al.*, 2011). Both proteins displayed higher mRNA levels at 6 hpi (which largely represents recently invaded tachyzoites) and at 56 hpi (representing tachyzoites shortly prior to or already undergoing egress). Lower mRNA levels were measured at 24 hpi (a time point representing early exponential replication). Subsequently, once exponential growth of parasites was almost completed (48 hpi), mRNA levels of Nc*ROP40* and Nc*ROP2Fam-1* gradually increased to reach again their highest value. According to our findings, developmental transitions in *Plasmodium falciparum* and *T. gondii* have shown to be strongly influenced by changes in mRNA levels (Radke *et al.*, 2005; Le Roch *et al.*, 2004). Indeed, a modal switch from expression of proteins involved in invasion and motility has been also described in extracellular tachyzoites of *T. gondii* (Gaji *et al.*, 2011; Lescault *et al.*, 2010). This could suggest that Nc*ROP40* and Nc*ROP2Fam-1* proteins are required for the subsequent phases of the lytic cycle in which both are highly transcribed. This phenomenon is consistent with the "just-in-time" concept stated for *P. falciparum* and *T. gondii*, whereby gene expression is only activated as their biological function becomes necessary to the parasite

(Behnke *et al.*, 2010; Radke *et al.*, 2005; Llinas & DeRisi, 2004).

In addition to monitoring mRNA levels during egress under normal culture conditions, the same was done by inducing egress artificially employing DTT at 48 hpi (Esposito *et al.*, 2007). DTT treatment induced a dramatic increase in Nc*ROP2Fam-1* expression after DTT supplementation, to levels similar to naturally occurring egress. Strikingly, and in contrast to Nc*ROP2Fam-1*, Nc*ROP40* mRNA levels were not substantially increased by DTT addition. To date, the mechanisms governing egress are not fully understood, but mounting evidence shows that intracellular calcium levels trigger the abrupt exit of parasites from PV, which is accompanied with a rapid decrease in host cell ATP (Blackman & Carruthers, 2013). Nc*ROP40* mRNA levels were unresponsive to the artificially-induced egress, suggesting that its up-regulation does not rely on the mechanisms triggering egress in contrast to Nc*ROP2Fam-1*.

Phosphorylation has a prominent key role in cellular regulatory processes and influences the functional activity of a plethora of enzymes and structural proteins. At 56 hpi, when the mRNA expression levels for Nc*ROP40* and Nc*ROP2Fam-1* reached a peak and tachyzoites were undergoing egress to infect another host cell, phosphorylation was evident in NcROP2Fam-1, but not in NcROP40. However, we cannot exclude that NcROP40 is phosphorylated at another phase of the lytic cycle. Predicted phosphorylation sites were found in NcROP40 and in NcROP2Fam-1. However, it is important to note that the phosphorylation prediction algorithms are optimized for mammalian cells or other cell types, and that rhoptry proteins are unique among eukaryotes and are only found in apicomplexan parasites. Thus, potential phosphorylation sites might not be accurately predicted. Previous works have shown that TgROP2 and TgROP4 are also phosphorylated, but only in intracellular parasites, indicating that phosphorylation is associated with protein regulation and its potential participation within the lytic cycle (Dunn *et al.*, 2008; Carey *et al.*, 2004). For NcROP2Fam-1, phosphorylation coincides with high mRNA levels, and since NcROP2Fam-1 was shown to be involved in host cell invasion (Alaeddine *et al.*, 2013), this could indicate that the protein is being activated to prepare tachyzoites for egress and/or invasion. To date, there is no information about the relevance of phosphorylation in NcROP proteins, and phosphorylation of all the known TgROP proteins has not been studied. Previous works suggested that phosphorylation of dense granule proteins has an influence on PVM association (Mercier *et al.*, 2005; Labruyere *et al.*, 1999). Thus, phosphorylation of NcROP2Fam-1 could be important for secretion and its subsequent association to the PVM, and this could be also applied to TgROP2 and TgROP4, both of which exhibit similar properties. However, further studies must be carried out in order to determine the role of rhoptry protein phosphorylation during the lytic cycle of *N. caninum* tachyzoites.

Pseudokinases are emerging as key regulators of cellular signaling (Reese *et al.*, 2014). Several studied rhoptry proteins in *T. gondii* have been described as kinases or pseudokinases, and some of them have shown the ability to remodel cellular transduction and the transcriptome of

the host cell through phosphorylation events (Jacot & Soldati-Favre, 2012; Lim *et al.*, 2012). Specifically, the TgROP18 and TgROP17 kinases and the TgROP5 pseudokinase form complexes and by that inactivate host immune responses and inflammation (Du *et al.*, 2014; Etheridge *et al.*, 2014). Moreover, TgROP16 regulates host innate immunity through STAT3 and STAT6 phosphorylation (Jensen *et al.*, 2013) and TgROP38 modulates MAPK signaling to control apoptosis and cell proliferation (Peixoto *et al.*, 2010). In our case, NcROP40 has been described as a predicted member of the rhoptry kinase family (ROPK) lacking the key kinase sequence motifs (Talevich & Kannan, 2013). The protein contains a structurally conserved N-terminal extension to the kinase domain that displays high sequence similarity to the NcROP5 and TgROP5 pseudokinases, among others. TgROP5 also lacks kinase activity (Reese & Boothroyd, 2011), but in contrast to NcROP40, is clearly secreted during invasion and associates with the PVM (El Hajj *et al.*, 2007a; El Hajj *et al.*, 2007b). Therefore, the role of NcROP40 as pseudokinase remains unclear. Nevertheless, the protein could be implicated in the regulation of still unknown virulence factors. Unfortunately, little is known about the existence of rhoptry virulence factors that could alter the host transcriptome after the infection with *N. caninum*. To date, the information about the orthologues of TgROP5, TgROP16, TgROP18 and TgROP38 in *N. caninum* is limited, and the only study in which they have been described is restricted to genomic and transcriptomic information that highlights the divergence of rhoptry proteins between *T. gondii* and *N. caninum* (Reid *et al.*, 2012). Hence, despite the common features of *N. caninum* and *T. gondii,* these distinct differences in their secreted virulence factors make it difficult to make direct extrapolations from one species to the other. However, the description of common mechanisms of the ROP2-family members required for the success of the lytic cycle and parasite proliferation could represent a valuable source for the development of novel vaccine candidates.

In summary, this study describes highly interesting features of the NcROP40 protein, and another member of the ROP2-family, NcROP2Fam-1, during the lytic cycle of *N. caninum* tachyzoites. Immunogold TEM clearly localized NcROP40 in the rhoptry bulbs of *N. caninum* tachyzoites but, in contrast to NcROP2Fam-1, we were unable to detect NcROP40 secretion into the host cell, which is likely an effect of the protein dilution within the host cytosol. mRNA quantification showed that Nc*ROP40* is highly expressed during egress and invasion, although its mRNA levels were not affected when egress was induced by DTT supplementation. These findings suggest differences in the transcriptional regulation and functional role of NcROP40 and NcROP2Fam-1. In addition, no evidence was found for NcROP40 phosphorylation at the time point of egress, in contrast to NcROP2Fam-1. NcROP40, together with NcROP2Fam-1, is a promising vaccine candidate, thus further studies will be carried out in order to elucidate its functionality. Epitope-tag assays and generation of Δ*rop40* knockout parasites would be useful to confirm more accurately whether NcROP40 is secreted or not, and to establish the role of the NcROP40 protein within the lytic cycle of *N. caninum*.

Acknowledgements

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Author contributions

JRC, GAG and LMOM conceived and designed the experiments. IPF, EJR, VMH and AH performed the experiments. IPF, JRC, GAG and LMOM analyzed the data. IPF, JRC, EJR, GAG, VMH, AH and LMOM wrote the paper.

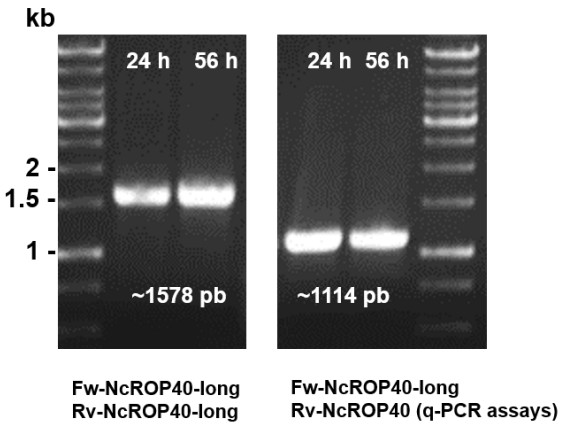
Supplementary Table 1: Primers used for Nc*ROP40* sequencing among three *N. caninum* isolates of different origins (Nc-Liv, Nc-Spain7 and Nc-Spain1H).

Primer	Sequences	Reference
Fw-chrV_ROP40	5'-TAAGAACGCATGGCTGACTG-3'	This study
Rv-chrV_ROP40	5'-CTGACAACGGCTCCTCTTTC-3'	This study
Fw-ROP40	5'-CGAGCTCATGGTGAAATCCCTGCACAAG-3'	Regidor-Cerrillo *et al*., 2012
Rv-ROP40	5'-CCTTAATTAATCACCCCACCACTGAACGC-3'	Regidor-Cerrillo *et al*., 2012
Rv-ROP40-int	5'-CTTCTGGCTTTTGCTGCTCC-3'	This study
Fw-ROP40-int	5'-ACGCACTCTCTTTGGCTTGT-3'	This study

Supplementary table 2: Primers used for Nc*ROP40,* Nc*ROP2Fam-1*, Nc*SAG1* and Nc*TUBα* cloning.

Protein	ToxoDB accession no.	Primer sequences	Reference	Length
Nc*ROP40*	NCLIV_012920	Fw-*Sac*I-CGAGCTCATGGTGAAATCCCTGCACAAG Rv-*Pac*I-CCTTAATTAATCACCCCACCACTGAACGC	Regidor-Cerrillo *et al*., 2012	1176 bp. 1-392 aa.
Nc*ROP2Fam-1*	NCLIV_001970	Fw-*Sac*I-GAGCTCTTGTGGCGTAATCAGAAGCAC Rv-*Hind*III-AAGCTTTTATAGCCTCGTGTCCTCCGT	Pastor-Fernández *et al*., 2015	1076 bp. 236-595 aa.
Nc*SAG1*	NCLIV_033230	Fw-CACTGGTGGCGTTCTTTGAC Rv-GCTATCGAGCCTACGAGTCC	This study	889 bp. 56-944 aa.
Nc*TUBα*	NCLIV_058890	Fw-GGTAACGCCTGCTGGGAG Rv-CTTCCTCTTCACCTTCGCCC	Alaeddine *et al*., 2013	1294 bp. 49-1342 aa.

Supplementary Figure 1: The Nc*ROP40*-long gene model was corroborated by reverse transcription PCR. mRNA was obtained from tachyzoites at 24 and 56 hours post-infection as described in the methods section. The whole Nc*ROP40*-long ORF was amplified from cDNA with the following primers: Fw-NcROP40-long (5'-ATGAGACACTCCTTGTGCTTTTC-3') and Rv-NcROP40-long (5'-TCACCCCACCACTGAACG-3'). In addition, the same forward primer was used with the reverse internal primer employed for the q-PCR assays (5'-TGGTGACTGCGACCAACTTA-3', from Table 1). In all the cases, PCR amplification yielded a single fragment with the expected molecular weight (see figure below).



Supplementary Figure 2: Sequence alignment of the coding region for the NcROP40 protein and its up and down-stream regions within the chromosome V of the Nc-Liv genome. NCLIV_chrV (662772-665947 position), NcROP40 and NcROP40-long are displayed as templates and were obtained from the ToxoDB source as described in methods section. Consensus sequences among Nc-Liv, Nc-Spain1H and Nc-Spain7 isolates were obtained by DNA sequencing and aligned based on the template sequences. Predicted aminoacidic sequence of the NcROP40-long protein is also displayed.

```
                         10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
>NCLIV_chrV         GTTTAACAGTCTCCAAATACTGCTTGATATGAAGGAAGATCTTCAGTTATGCTGCATTTC
>NcROP40            ------------------------------------------------------------
>NcROP40-long       ------------------------------------------------------------
Consensus NcLiv     ------------------------------------------------------------
Consensus NcSp7     ------------------------------------------------------------
Consensus NcSp1H    ------------------------------------------------------------
Aa.sequence         ------------------------------------------------------------

                         70        80        90       100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
>NCLIV_chrV         AATTGGCGGCGGTAGAGCCCAGGGGGAGCCTGCCTCATTTTAGGCCGGATGGCCGCGGTC
>NcROP40            ------------------------------------------------------------
>NcROP40-long       ------------------------------------------------------------
Consensus NcLiv     ------------------------------------------------------------
Consensus NcSp7     ------------------------------------------------------------
Consensus NcSp1H    ------------------------------------------------------------
Aa.sequence         ------------------------------------------------------------

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>NCLIV_chrV         AGACATTCTGTGCTAGTGCACCTCTTTCCGTATCTTGACTCCGCCAATACTCCCCAGCCC
>NcROP40            ------------------------------------------------------------
>NcROP40-long       ------------------------------------------------------------
Consensus NcLiv     ------------------------------------------------------------
Consensus NcSp7     ------------------------------------------------------------
Consensus NcSp1H    ------------------------------------------------------------
Aa.sequence         ------------------------------------------------------------

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>NcROP40            ------------------------------------------------------------
>NcROP40-long       ------------------------------------------------------------
Consensus NcLiv     ------------------------------------------------------------
Consensus NcSp7     ------------------------------------------------------------
Consensus NcSp1H    ------------------------------------------------------------
Aa.sequence         ------------------------------------------------------------

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>NCLIV_chrV         GCCTCTTTTTTAAGAACGCATGGCTGACTGCTAGCGCTGTTCAATCCGTTGACGTCGCGC
>NcROP40            ------------------------------------------------------------
>NcROP40-long       ------------------------------------------------------------
Consensus NcLiv     ------------------------------------------------------------
Consensus NcSp7     ------------------------------------------------------------GC
Consensus NcSp1H    ------------------------------------------------------------
Aa.sequence         ------------------------------------------------------------

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                    ....|....|....|....|....|....|....|....|....|....|....|....|
>NCLIV_chrV         AAGGTTGGGCTCTGGACAGATTCCGCCGCGCAGAATTGGCGCTAGTCTAGATCACAGACA
>NcROP40            ------------------------------------------------------------
>NcROP40-long       ------------------------------------------------------------
Consensus NcLiv     ----------------------------------AATTGGCGCTAGTCTAGATCACAGACA
Consensus NcSp7     AAGGTTGGGCTCTGGACAGATTCCGCCGCGCAGAATTGGCGCTAGTCTAGATCACAGACA
Consensus NcSp1H    ------------------------------------------------------------
Aa.sequence         ------------------------------------------------------------
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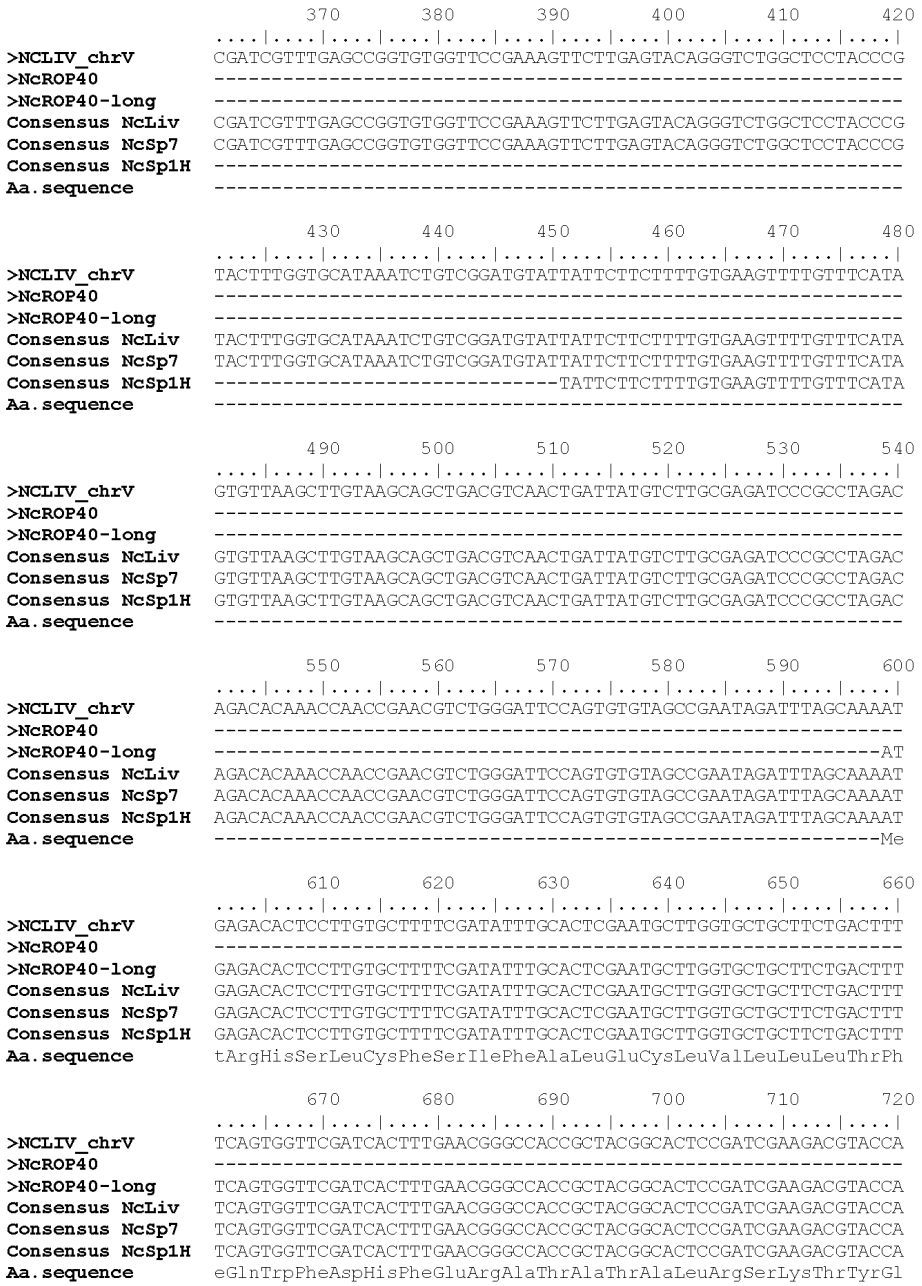
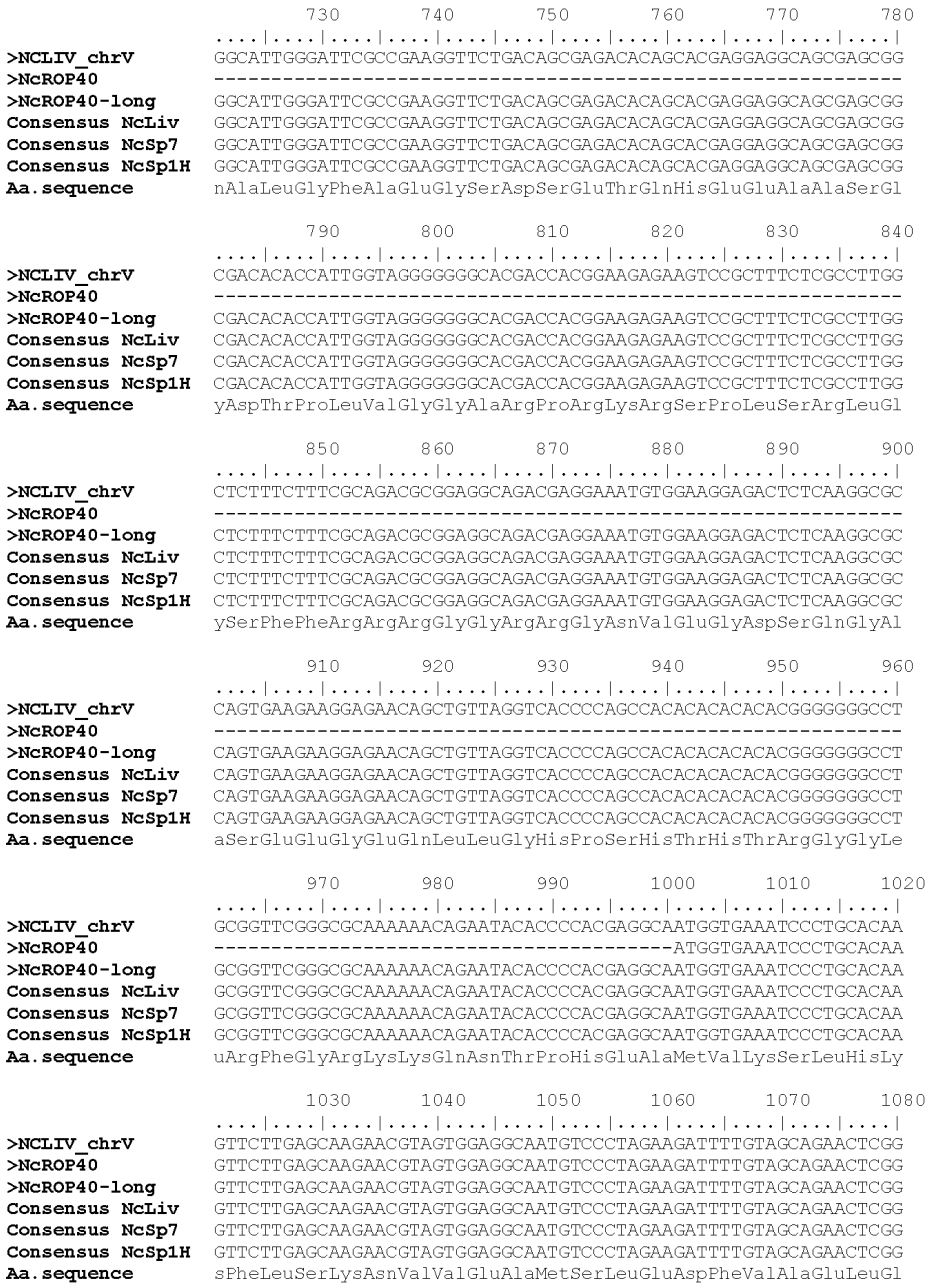
Supplementary Figure 2 (continuation)



Supplementary Figure 2 (continuation)



Supplementary Figure 2 (continuation)

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>NcROP40              TGTCGACCATCGTGCAATTCAACCTCCATTCTTTCGCAAGGGCGAGGGCGCGTCGAGAGC
>NcROP40-long         TGTCGACCATCGTGCAATTCAACCTCCATTCTTTCGCAAGGGCGAGGGCGCGTCGAGAGC
Consensus NcLiv       TGTCGACCATCGTGCAATTCAACCTCCATTCTTTCGCAAGGGCGAGGGCGCGTCGAGAGC
Consensus NcSp7       TGTCGACCATCGTGCAATTCAACCTCCATTCTTTCGCAAGGGCGAGGGCGCGTCGAGAGC
Consensus NcSp1H      TGTCGACCATCGTGCAATTCAACCTCCATTCTTTCGCAAGGGCGAGGGCGCGTCGAGAGC
Aa.sequence           yValAspHisArgAlaIleGlnProProPhePheArgLysGlyGluGlyAlaSerArgAl

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>NcROP40              TGTCGGGTATTTCGCGGAGCAGCAAAAGCCAGAAGTATCTGAAGAGACGCGGAAAACTTT
>NcROP40-long         TGTCGGGTATTTCGCGGAGCAGCAAAAGCCAGAAGTATCTGAAGAGACGCGGAAAACTTT
Consensus NcLiv       TGTCGGGTATTTCGCGGAGCAGCAAAAGCCAGAAGTATCTGAAGAGACGCGGAAAACTTT
Consensus NcSp7       TGTCGGGTATTTCGCGGAGCAGCAAAAGCCAGAAGTATCTGAAGAGACGCGGAAAACTTT
Consensus NcSp1H      TGTCGGGTATTTCGCGGAGCAGCAAAAGCCAGAAGTATCTGAAGAGACGCGGAAAACTTT
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>NcROP40              GGAAGCGGTTGAACCAGTTCTGCCTGCCGGACAGCCACTGTCTTTTCACACTACATACGA
>NcROP40-long         GGAAGCGGTTGAACCAGTTCTGCCTGCCGGACAGCCACTGTCTTTTCACACTACATACGA
Consensus NcLiv       GGAAGCGGTTGAACCAGTTCTGCCTGCCGGACAGCCACTGTCTTTTCACACTACATACGA
Consensus NcSp7       GGAAGCGGTTGAACCAGTTCTGCCTGCCGGACAGCCACTGTCTTTTCACACTACATACGA
Consensus NcSp1H      GGAAGCGGTTGAACCAGTTCTGCCTGCCGGACAGCCACTGTCTTTTCACACTACATACGA
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Consensus NcSp7       CAGAAAAGGCTCTTACTTCAAAAGAGGCAGCTTGTTTCACCGAGATTTCTTCGAGTTCTT
Consensus NcSp1H      CAGAAAAGGCTCTTACTTCAAAAGAGGCAGCTTGTTTCACCGAGATTTCTTCGAGTTCTT
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Consensus NcLiv       CATTGATGGGCAACCTTTTGATCTGAGGATACTCCCACTACCGAGCGGTGAAGAGGGGGA
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Consensus NcSp1H      AGCTACGCTGGAACGATACAAAAAGGAGCTGGAAAATGAGCGGAGTGTTCGACTTCAATT
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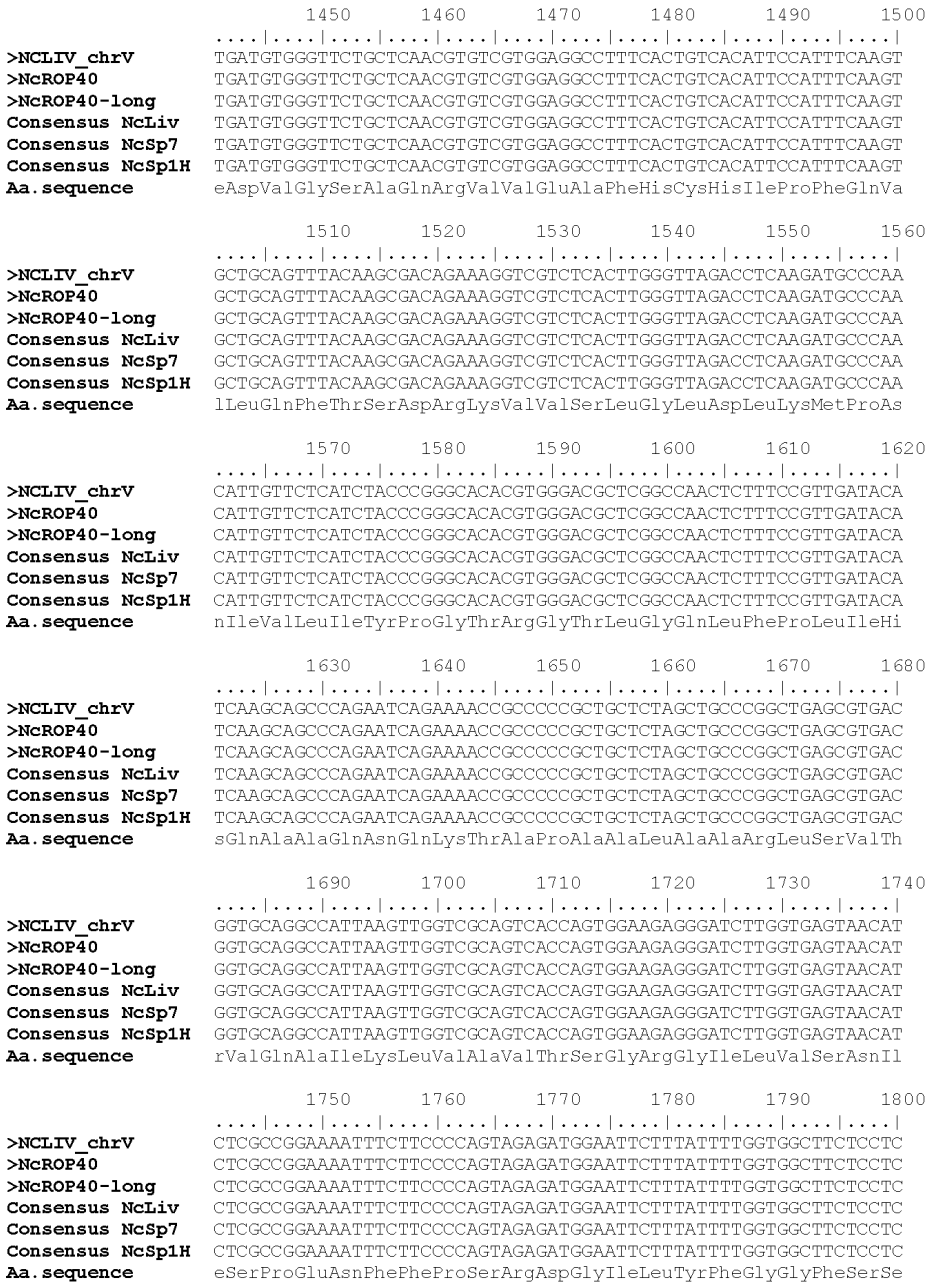
Supplementary Figure 2 (continuation)



Supplementary Figure 2 (continuation)

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Consensus NcSp7         GCCGAACGTGACTTCCAGGGGGAGACGGTTCACCGCTGAAGACAACGCAGCAGACTTAGG
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Consensus NcSp7         ACGCACTCTCTTTGGCTTGTGGTGCGGCGGTTCGCTTCCTGAGGATGAACCGAGTGGGCG
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Consensus NcSp7         GGCCGACGTGGATTTCTCCAATTGCGGGACAGACCTGCCGGATCCTGTCAAGAAGTTAAT
Consensus NcSp1H        GGCCGACGTGGATTTCTCCAATTGCGGGACAGACCTGCCGGATCCTGTCAAGAAGTTAAT
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Consensus NcSp7         TATGGGAGTGTCCGGCTCCCACGGACGCGCCCCTCTCAGCGCCTCCCAGGTCCTCGATAC
Consensus NcSp1H        TATGGGAGTGTCCGGCTCCCACGGACGCGCCCCTCTCAGCGCCTCCCAGGTCCTCGATAC
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                        2110      2120      2130      2140      2150      2160
                        ....|....|....|....|....|....|....|....|....|....|....|
>NCLIV_chrV             TCCAAACTATCAGGAGCTGCGCAGATTAGAAAAAGAAGTGTCTCAGAGTGTCGCGGTGCG
>NcROP40                TCCAAACTATCAGGAGCTGCGCAGATTAGAAAAAGAAGTGTCTCAGAGTGTCGCGGTGCG
>NcROP40-long           TCCAAACTATCAGGAGCTGCGCAGATTAGAAAAAGAAGTGTCTCAGAGTGTCGCGGTGCG
Consensus NcLiv         TCCAAACTATCAGGAGCTGCGCAGATTAGAAAAAGAAGTGTCTCAGAGTGTCGCGGTGCG
Consensus NcSp7         TCCAAACTATCAGGAGCTGCGCAGATTAGAAAAAGAAGTGTCTCAGAGTGTCGCGGTGCG
Consensus NcSp1H        TCCAAACTATCAGGAGCTGCGCAGATTAGAAAAAGAAGTGTCTCAGAGTGTCGCGGTGCG
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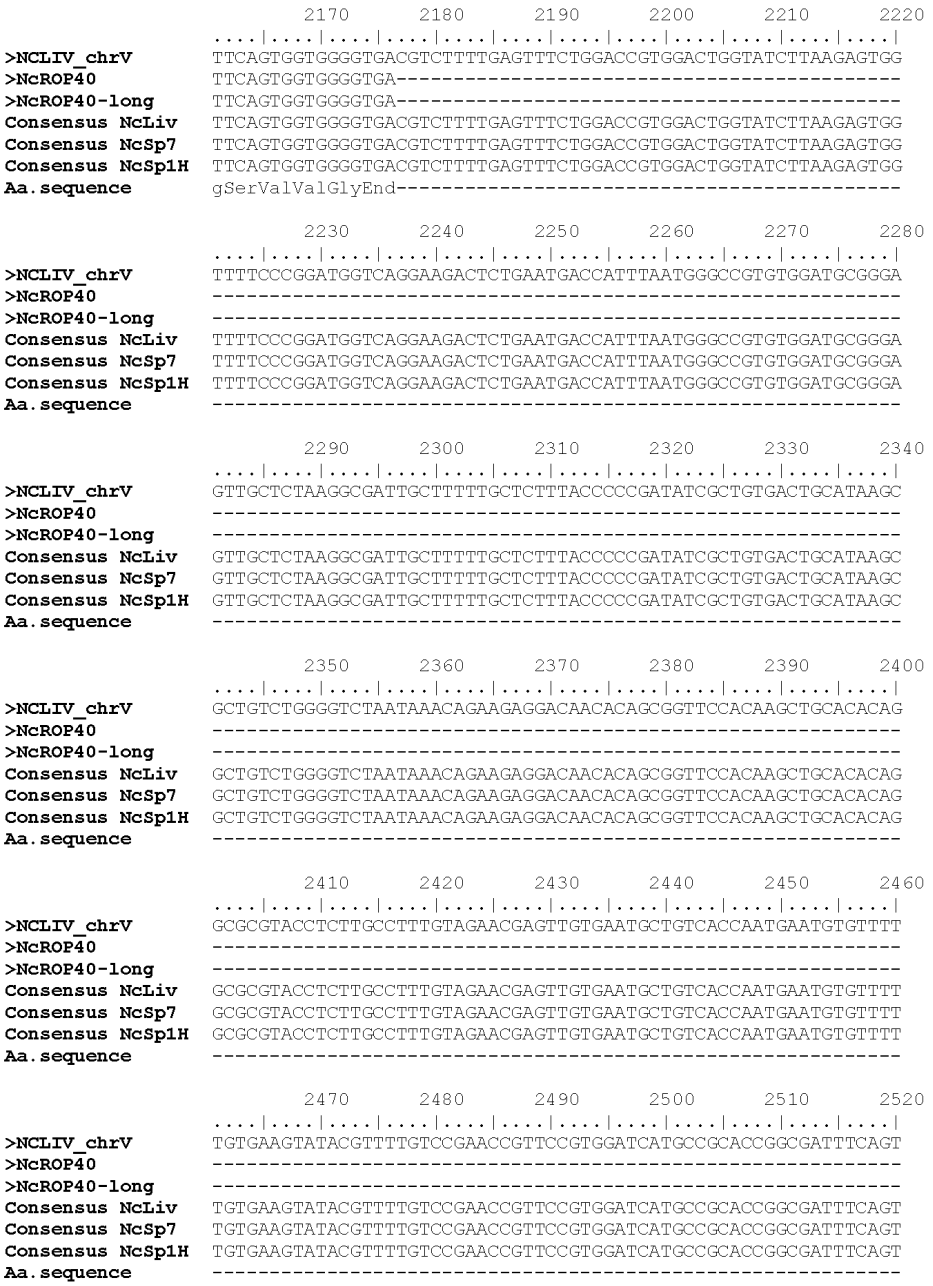
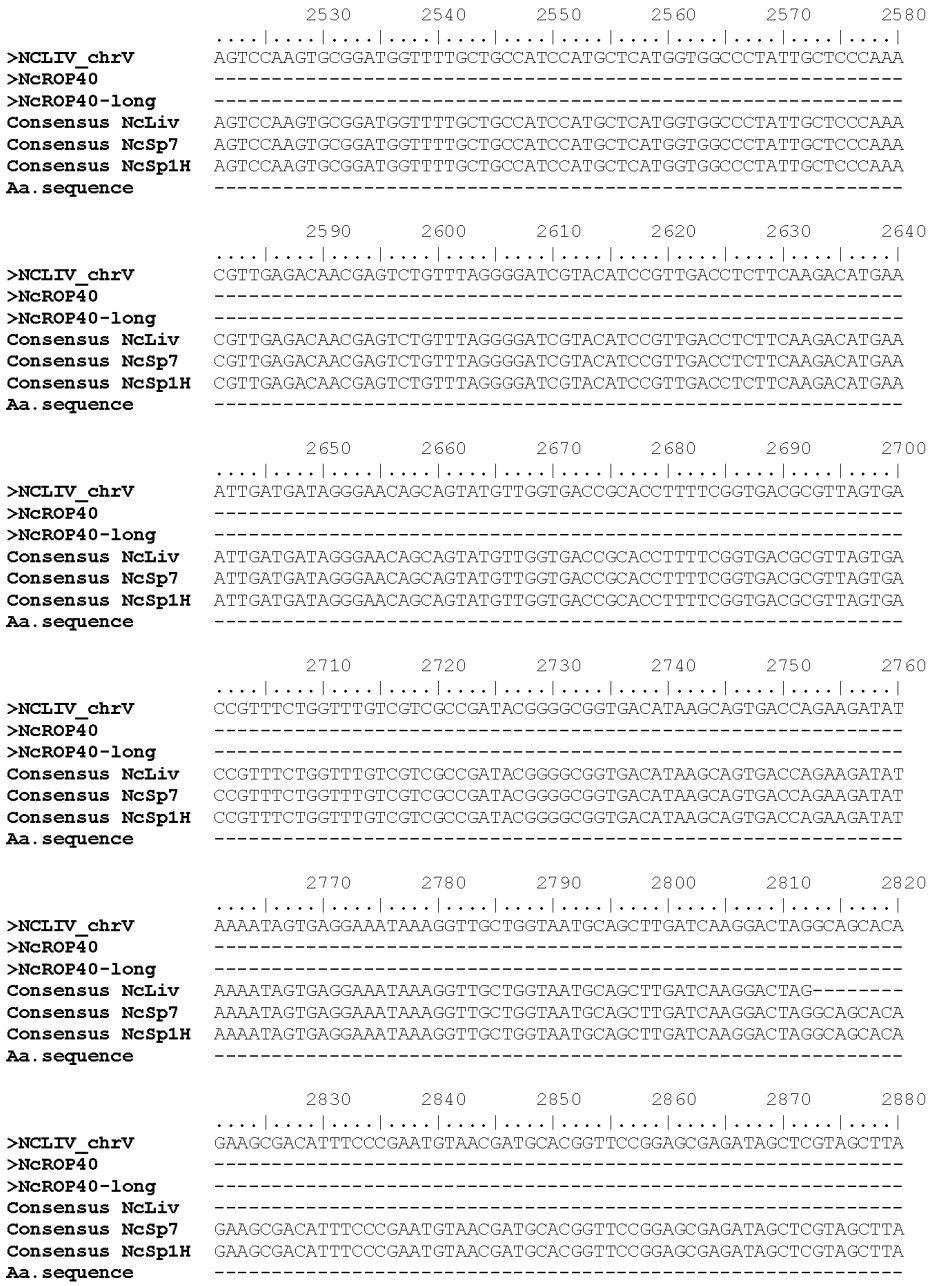
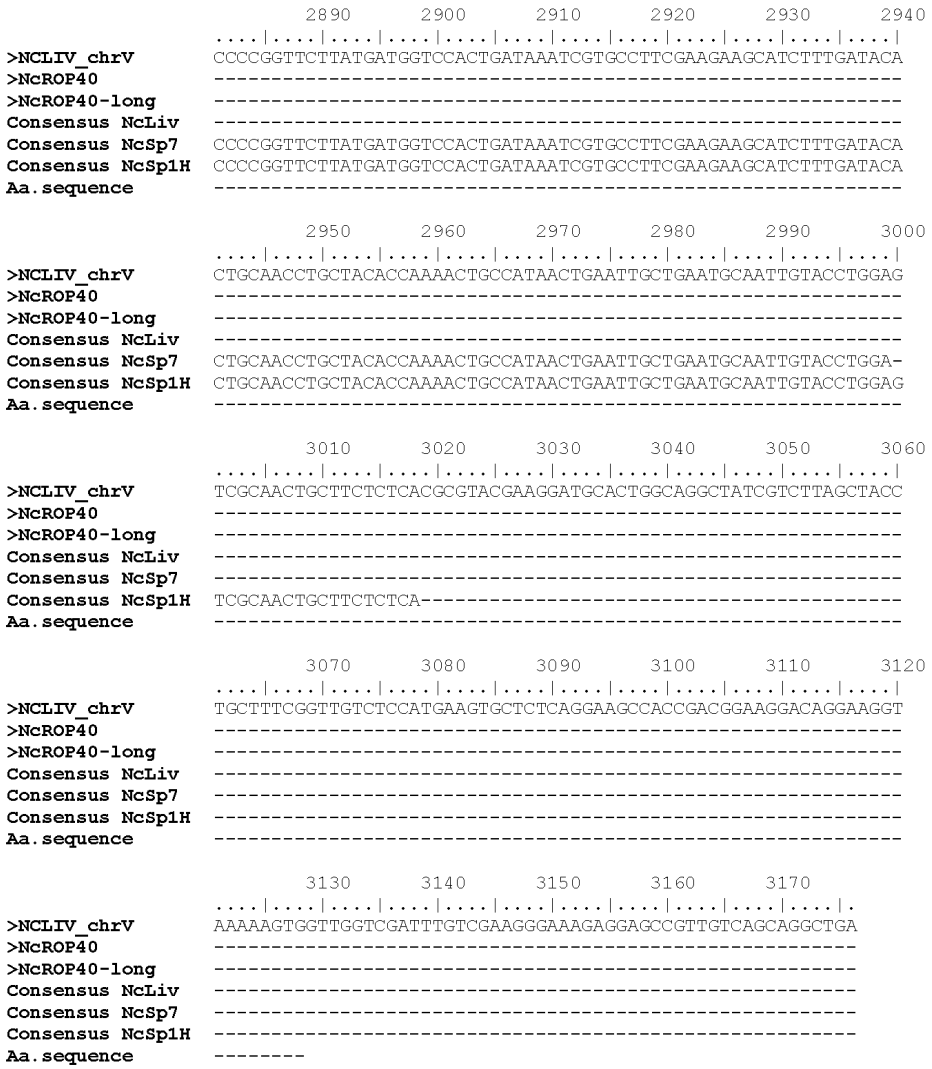
Supplementary Figure 2 (continuation)



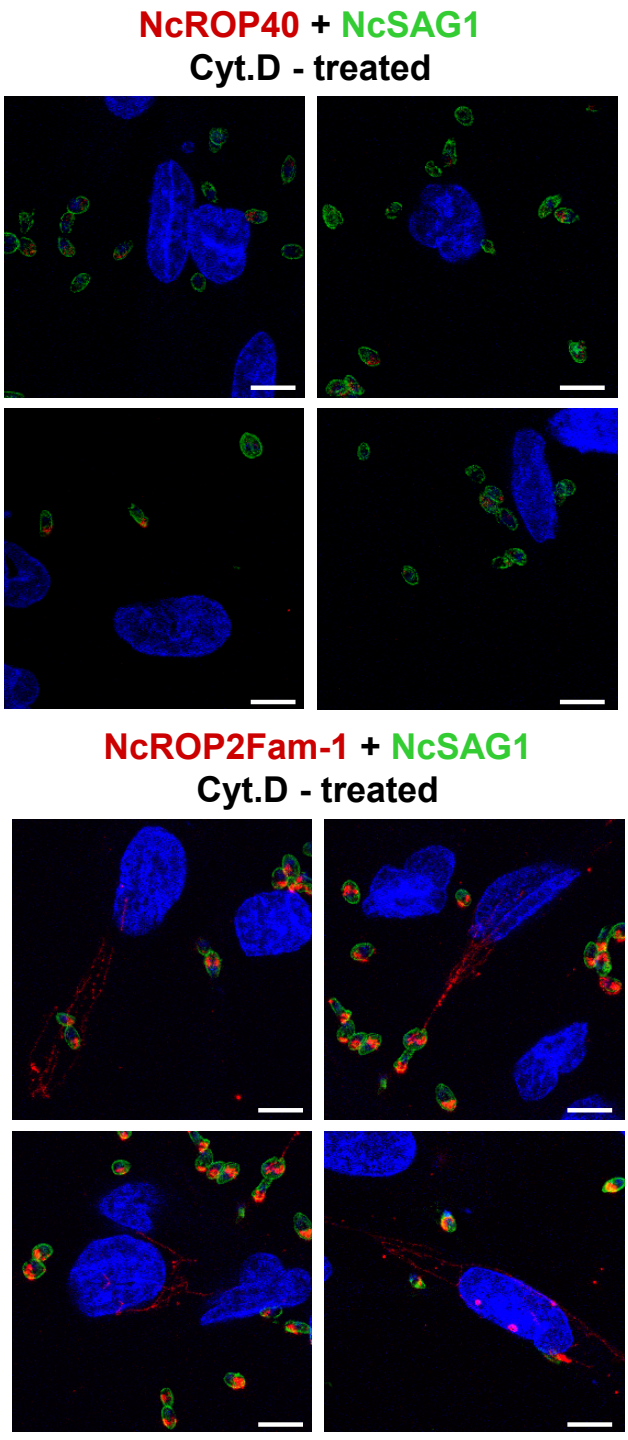
Supplementary Figure 2 (continuation)



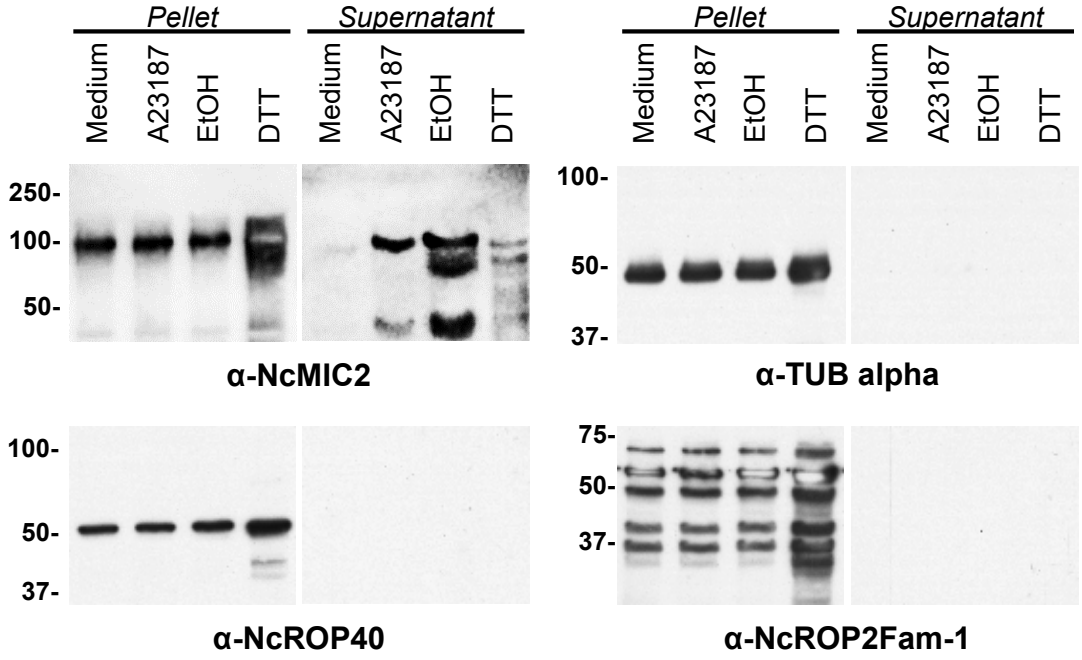
Supplementary Figure 2 (continuation)



Supplementary Figure 3: Determination of NcROP40 and NcROP2Fam-1 secretion by evacuole assays. These experiments were carried out as described previously by Dunn *et al.*, (2008), by incubating cytochalasin D-treated tachyzoites with human foreskin fibroblasts (HFFs) for 1 h prior to fixation. Evacuoles were detected by confocal laser-scanning microscopy using the affinity purified PAbs α-NcROP40 (red, left) and α-NcROP2Fam-1 (red, right). The MAb α-NcSAG1 (green) was employed as a surface marker. Only NcROP2Fam-1 was detected in evacuoles, whilst NcROP40 release was not observed. Images represent three merged stacks of 1 µm each. Bars represent 1 µm.



Supplementary Figure 4: Effect of A23187, ethanol and DTT on secretion of NcROP40 and NcROP2Fam-1 proteins as shown by Western-blot using respective antibodies. The same protein samples were also probed by immunoblotting with α-NcMIC2 and α-TUBα antibodies to (i) confirm induced secretion and (ii) exclude inadvertent tachyzoite lysis, respectively. Rhoptry discharge was not observed in culture supernatants upon any of these treatments, whilst NcMIC2 secretion was evident after A23187, ethanol and DTT supplementation. Tachyzoite lysis was not detected. All the antibodies specifically reacted against their respective protein on tachyzoite extracts.



The tandemly repeated NTPase (NTPDase) from *Neospora caninum* is a canonical GRA protein whose expression, secretion and phosphorylation coincides with the tachyzoite egress

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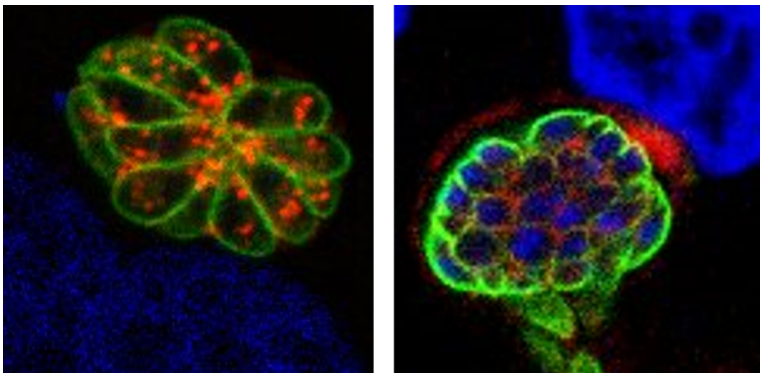

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ABSTRACT

NTPases (also NTPDases) are enzymes with apyrase activity. They are widely distributed among eukaryotes, and also within the Sarcocystidae family members. In *Toxoplasma gondii,* the TgNTPase accumulates in the dense granules, and has been associated with the strain virulence. In the closely related *Neospora caninum*, the NcNTPase appears to be more abundant in two virulent isolates compared with an attenuated one, indicating that it contributes to the pathogenicity of neosporosis. However, so far no additional information on NcNTPase has been provided. We here characterize the NcNTPase in comparison with the previously described dense granule protein NcGRA7. In addition, we describe the presence of three different *loci* containing three copies of the Nc*NTPase* within the Nc-Liv genome, and report the existence of up to six different Nc*NTPase* alleles. We also provide evidence for the occurrence of diverse species of the NcNTPase at the protein level. Both NcNTPase and NcGRA7 were similarly up-regulated, secreted, and phosphorylated during the egress and/or early invasion phases. However, in contrast to NcGRA7, secretion of NcNTPase was not affected by the addition of calcium modulators such as A23187 and ethanol, suggesting that these two proteins exhibit differential regulation of secretion. The present study suggests that NcNTPase and NcGRA7 may be essential for progression through the lytic cycle. Nevertheless, whether the expression of certain NcNTPase isoforms is associated with parasite virulence remains to be elucidated.

1. Introduction

Neospora caninum is an apicomplexan cyst-forming parasite that causes abortion in cattle and neuromuscular disorders in canids. Rapidly replicating tachyzoites are responsible for parasite dissemination and harmful effects within the infected host, resulting in vertical transmission and abortion (Dubey & Schares, 2011). Host tissue damage occurs as a consequence of the tachyzoite lytic cycle, a tightly regulated process that enables parasite propagation with devastating effects for the infected cells (Hemphill *et al.*, 2004; Hemphill *et al.*, 1996). The lytic cycle has been extensively studied in the closely related parasite *Toxoplasma gondii* (Sheiner & Soldati-Favre, 2008; Black & Boothroyd, 2000), but only scarcerly investigated in *N. caninum*. However, preliminary studies showed that the molecular mechanisms of this process are highly conserved among *T. gondii* and *N. caninum* (Hemphill *et al.*, 2013).

Micronemes, rhoptries, and dense granules are secretory organelles exclusively found in apicomplexan parasites. The contents of these organelles are sequentially released during the lytic cycle, and play a crucial role in the host-parasite interactions. Specifically, dense granule (GRA) proteins are secreted into the parasitophorous vacuole (PV), a metabolically active compartment designed to favour parasite replication, and modify the PV membrane (PVM) to render it resistant to fusion with host lysosomes (Beyer *et al.*, 2002; Mercier *et al.*, 2002). More than 20 GRA proteins have been reported for *T. gondii* (Mercier & Cesbron-Delauw, 2015), and 15 have been identified in *N. caninum* at protein and transcriptional levels (Aguado-Martínez *et al.*, 2010; Guionaud *et al.*,

2010)(Reid *et al.*, 2012). Nevertheless, only a limited number of GRA proteins have been studied in *N. caninum*, despite the fact that some GRA proteins such as TgGRA7, TgGRA15, TgGRA16, TgGRA24, TgGRA25 and TgNTPase I, among others, have been associated with virulence mechanisms in *T. gondii* (Hermanns *et al.*, 2015; Shastri *et al.*, 2014; Braun *et al.*, 2013; Rosowski *et al.*, 2011; Asai *et al.*, 1995). In fact, TgNTPase and the TgGRA7 are essential for the replication of *T. gondii* tachyzoites (Cesbron-Delauw *et al.*, 2008; Coppens *et al.*, 1999).

The *N. caninum* GRA7 protein was extensively characterized during the last years (Aguado-Martínez *et al.*, 2010; Álvarez-García *et al.*, 2007; Hemphill *et al.*, 1998), whereas little information is available for the NcNTPase. This protein appears to be more abundantly expressed in virulent isolates, suggesting that its function could be related with parasite virulence (Regidor-Cerrillo *et al.*, 2012). Besides, multiple genes coding for NTPase have been identified in both, *N. caninum* and *T. gondii* (Asai *et al.*, 1998; Bermudes *et al.*, 1994). In fact, *T. gondii* tachyzoites express two NTPase isoforms (NTPase 1 and 3, also termed NTPase II and I, respectively), which differ in their enzymatic activities, although the TgNTPase 3 is restricted to the virulent type I strains (Krug *et al.*, 2012; Asai *et al.*, 1995). In contrast, only NcNTPase 3 (or NcNTPase I) activity has been found in tachyzoite extracts of *N. caninum* (Asai *et al.*, 1998), and how it contributes to parasite virulence is still unknown.

We here present a comparative analysis of NcNTPase and NcGRA7 in terms of protein dynamics, secretion, phosphorylation, and mRNA expression profiles during the tachyzoite lytic cycle. This study increases the limited existing knowledge on these GRA proteins in *N. caninum*, and will serve as reference for future studies intended to establish their functional role during the proliferative phase of *N. caninum.*

2. Materials and methods

2.1. In silico sequence analysis

All sequence data were obtained from ToxoDB v24 (www.toxodb.org) and edited using the BioEdit software v7.1.1. BLAST tool from the NCBI website (www.ncbi.nlm.nih.gov/BLAST/) was employed to match homologous sequences. Protein families were acquired from Pfam database (pfam.sanger.ac.uk/, Sanger).

2.2. Parasite culture

The Nc-Liv isolate (Barber *et al.*, 1995) was propagated *in vitro* by continuous passage in MARC-145 cell culture using standard procedures (Pérez-Zaballos *et al.*, 2005). For transmission electron microscopy (TEM), murine epidermal keratinocyte cultures were infected with the Nc-Liv isolate as described earlier (Vonlaufen *et al.*, 2002).

2.3. Production of recombinant proteins and polyclonal antibodies

rNcNTPase and rNcGRA7 proteins were obtained as previously described (Pastor-Fernández *et al.*, 2015a). Briefly, proteins were cloned in the pET45b(+) expression system (Novagen), expressed in *E. coli* BL21(DE3) pLysS competent cells (Agilent Technologies) as a (His)6-tagged fusion proteins, and purified using HisTrapHP columns coupled to the ÄKTAprime Plus system (GE Healthcare). All proteins were

analysed by SDS-PAGE to check their purity and integrity. Electrophoresed proteins were manually excised from prepared Coomassie-stained 1-D gels for peptide mass fingerprint (MS) following standard procedures (Risco-Castillo *et al.*, 2007).

Polyclonal antibodies (PAbs) against rNcNTPase were raised in two female New Zealand White rabbits as previously described (Pastor-Fernández *et al.*, 2015b). Affinity purified antibodies were prepared following standard procedures (Álvarez-García *et al.*, 2007). All protocols followed the proceedings detailed by the current legislation at the time of the experiment (Spanish Royal Decree 1201/2005) and were approved by the Animal Research Committee of the Complutense University. Polyclonal and monoclonal antibodies (MAbs) against rNcGRA7 were obtained as previously described (Aguado-Martínez *et al.*, 2010; Álvarez-García *et al.*, 2007).

2.4. 1-DE and 2-DE immunoblot

The NcNTPase protein was detected on Nc-Liv parasite extracts by 1-DE Western-Blot as previously described (Pastor-Fernández *et al.*, 2015b). PAbs α-rNcNTPase were used as primary antibody, whereas goat anti-rabbit IgG antibody conjugated to peroxidase (Sigma-Aldrich) was used as secondary antibody. Both antibodies were diluted at 1:1,000. Reactions were developed using 4-chloro-1-naphtol (Bio-Rad) as substrate until signal visualization.

For 2-DE immunoblot Nc-Liv tachyzoites were purified in desalting columns and protein extraction was performed as previously described (Regidor-Cerrillo *et al.*, 2015). Briefly, 2×10^8 frozen tachyzoites were resuspended in 200 µl of lysis buffer (6 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTE, 10 mM Tris-HCl and 1 mM PMSF), processed by 3 cycles of freezing and thawing and solubilized by the addition of 200 µl of rehydratation buffer (8 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTE, and 1% ampholyte). Insoluble material was removed by centrifugation at 13,000 rpm for 30 min at 4 ºC and protein concentration of the resulting supernatants were determined by the Bradford method (Bio-Rad) using bovine serum albumin as standard. Protein extracts (100 µg) were resolved by 2DE-SDS PAGE as previously described (Regidor-Cerrillo *et al.*, 2015). Isoelectric focusing (IEF) was performed on 17cm-ReadyStrip™ IPG Strips pH 3–10 NL (Bio-Rad) with a Protean IEF cell system (Bio-Rad). Before the second-dimension separation, proteins on the strips were reduced with 4% DTE and then alkylated with 5% iodoacetamide in equilibration buffer (6 M urea, 50 mM Tris–HCl pH 6.5, 30% glycerol, and 2% SDS). Second-dimension electrophoresis and immunoblotting was performed as previously described (Regidor-Cerrillo *et al.*, 2015). After 2-DE SDS–PAGE, gels were transferred at 18 ºC onto PVDF membranes for immunoblotting. The blotted membrane was blocked with TBS-Tween 20 buffer containing 5% (w/v) dry milk and incubated with the PAb α-rNcNTPase at a 1:50,000 dilution. Then, the membranes were incubated with a goat anti-rabbit IgG antibody conjugated to peroxidase at a 1:100,000 dilution (Sigma-Aldrich). Blots were exposed for 1–30 sec using the Immobilon Western Chemi-luminescent HRP Substrate (Millipore). AGFA CP1000 processor and AGFA films (Curix/RP2 Plus, 18 cm × 24 cm) were used for image

acquisition. Image capturing was performed using the PDQuest™ (Bio-Rad) program.

2.5. Transmission electron microscopy (TEM)

TEM experiments were carried out as previously described (Risco-Castillo *et al.*, 2007). Infected keratinocyte cultures were fixed, LR-White embedded and labeled with affinity-purified rabbit α-rNcNTPase at a dilution of 1:2, and goat anti-rabbit conjugated to 10 nm diameter gold particles diluted at 1:5, both in PBS / 0.3% BSA (Amersham). After extensive washing in PBS, the grids were air-dried, and then contrasted with uranyle acetate and lead citrate (Hemphill *et al.*, 2004). Specimens were viewed on a Phillips 600 TEM operating at 60 kV.

2.6. Secretion assays

Secretion assays were performed as previously described (Pastor-Fernández *et al.*, 2015b). Briefly, 1×10^8 tachyzoites were suspended in 500 µl cold phenol red-free DMEM and stimulated with 10 µM A23187 (Sigma-Aldrich), 1% ethanol (Merck Chemicals), or 10 mM dithiothreitol (DTT, Calbiochem) for 20 minutes at 37º C. Non-stimulated parasites were kept on ice. Secretory fractions in the supernatants were recovered by centrifugation, filtered and supplemented with phosphatase and protease inhibitor cocktails (Sigma-Aldrich). Pelleted parasites were resuspended in cold PBS, supplemented with phosphatase and protease inhibitor cocktails, and recovered by centrifugation. All samples were stored at -80º C until further analyses.

Pellets were resuspended in 1× Laemmli sample buffer, and supernatans were dissolved in 5× Laemmli sample buffer to the same final volume (600 µl). In order to estimate protein secretion, equal amounts of secretion supernatants and tachyzoite lysates (20 µl/lane, ~3×10^6 tachyzoites) were loaded onto SDS-PAGE gels for immunoblot analyses. Detection of NcTUBα in secretion supernatants was employed as tachyzoite lysis indicator, whilst NcMIC2 detection was used as positive control of secretion (Lovett *et al.*, 2000). PVDF membranes were incubated with α-rNcNTPase, α-rNcGRA7 and α-rNcMIC2 at a dilution of 1:5,000, and with α-TUBα at a 1:10,000 dilution (MAb, Sigma-Aldrich). Goat anti-rabbit IgG and goat anti-mouse IgG antibody conjugated to peroxidase were employed as secondary antibodies at 1:25,000 and 1:80,000 dilution, respectively (Sigma-Aldrich). Reactions were developed by chemiluminiscence as described above.

2.7. Protein dynamics throughout the lytic cycle by immunofluorescence

The localization of NcNTPase and NcGRA7 proteins during the lytic cycle of *N. caninum* tachyzoites from 1 to 56 hours post-infection (hpi) was studied by immunofluorescence following previously described protocols (Pastor-Fernández *et al.*, 2015b). Ice-cold methanol, 2% paraformaldehyde in PBS, or 2% paraformaldehyde-0.05% glutaraldehyde in PBS, were used as fixatives for 10 to 30 minutes. Blocked and permeabilised coverslips were labeled with MAb α-NcSAG1 as a tachyzoite surface marker (1:250 dilution) (Björkman & Hemphill, 1998), and subsequently with affinity purified PAbs against rNcNTPase and rNcGRA7 (1:8 dilution). Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes) were

employed as secondary antibodies at a 1:1,000 dilution. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) dye and coverslips were mounted on glass slides with ProLong® Gold antifade reagent (Molecular Probes) for the microscopic visualization.

In addition, in order to co-localise NcGRA7 and NcNTPase proteins, some coverslips were incubated with MAb α-NcGRA7 (1:25 dilution) and affinity purified PAb α-NcNTPase (1:8 dilution), and then with the Alexa Fluor 594-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG at 1:1,000 dilution, respectively.

Single 1µm slices of immunofluorescence stainings were captured with a Leica TCS-SPE confocal laser-scanning microscope (Leica Microsystems) in the Department of Biochemistry and Molecular Biology IV of the Complutense University (Madrid). Image processing was performed using the LAS AF (Leica Microsystems) and the ImageJ software (NCBI, http://rsb.info.nih.gov/ij/).

2.8. Evaluation of NcNTPase and NcGRA7 mRNA expression levels

Messenger RNA levels during the tachyzoite lytic cycle were determined by real-time reverse transcription PCR following a previously described protocol (Pastor-Fernández *et al.*, 2015b). Samples were obtained at four representative time points, representing either recent invasion, PV maturation, exponential growth of parasites or tachyzoite egress. For this purpose, MARC-145 cultures were infected with the Nc-Liv isolate and maintained during 6, 24, 48 and 56 hours. The effect of induced egress at 48 hpi with 10 mM DTT on mRNA levels of Nc*GRA7* and Nc*NTPase* was also studied. Total RNA was extracted using Maxwell® 16 LEV simplyRNA Purification Kit and RNA integrity was checked by electrophoresis on agarose gels. Reverse transcription was carried out with the master mix SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) and resulting cDNA was diluted 1:20 and analysed by real-time PCR using the Power SYBR® Green PCR Master Mix (Applied Biosystems) in the ABI 7300 Real Time PCR System (Applied Biosystems). Primers used for amplification of Nc*NTPase*, Nc*GRA7* and the housekeeping genes Nc*Tubulin alpha* (*TUBα*) and Nc*SAG1* are shown in Table 1. Plasmids containing DNA targets for real-time PCR were employed as standard curves (pET45b(+)-Nc*GRA7*, pET45b(+)-Nc*NTPase*, pGEM-T-Nc*SAG1*, and pGEM-T-Nc*TUBα*) (Pastor-Fernández *et al.*, 2015b). A seven-point duplicate standard curve based on 10-fold serial dilutions was included on each run. The -ΔCt values were calculated by subtracting the Ct value of the normalizer genes from the Ct value of each sample. Relative fold increases or decreases were assessed by the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008) using the mean expression values at 24 hours post-infection as baseline. Raw RNA samples were included in each batch of amplifications to confirm the absence of *N. caninum* genomic DNA. Data analyses of mRNA levels were carried out by Kruskal-Wallis and Dunn's tests using GraphPad Prism v.6.01 software.

Table 1: Primers used to amplify Nc*NTPase*, Nc*GRA7*, Nc*SAG1* and Nc*TUB*α sequences by real time-PCR.

Protein	ToxoDB accession no.	Primer sequences	Reference	Length	Introns [a]	Slope [b]	R^2 [b]
Nc*NTPase*	NCLIV_068400	ATTGACCCCGACAGTATTCG ACGCTTGGAATCAACAGACCT	This study	129 bp. Pos. 283-411	No	-3.54	0.998
Nc*GRA7*	NCLIV_021640	GAACAGCATGAAGGGGACAT CACCATCTGTAATGGCATCG	This study	130 bp. Pos. 97-226	No	-3.61	0.997
Nc*SAG1*	NCLIV_033230	CGGTGTCGCAATGTGCTCTT ACGGTCGTCCCAGAACAAAC	(Fernández-García *et al.*, 2006)	150 bp. Pos. 504-653	No	-3.24	0.997
Nc*TUBα*	NCLIV_058890	GGTAACGCCTGCTGGGAG GCTCCAAATCCAAGAAGACGCA	(Alaeddine *et al.*, 2013)	166 bp. Pos. 49-214	Yes*	-3.24	0.994

[a] Primers for intron-containing sequences were designed using cDNA as template. * Forward primer for Nc*TUBα* amplification annealed at intron splice junction to prevent amplification of genomic DNA.

[b] Descriptive values of real time-PCR from standard curves for each pair of primers are shown.

2.9. Phosphorylation assays

Phosphorylation assays were performed using *N. caninum* infected MARC-145 cells at 56 hours post-infection (Pastor-Fernández *et al.*, 2015b). Cell cultures were resuspended in alkaline phosphatase-compatible buffer (100 mM sodium chloride [Panreac], 50 mM Tris-HCl [Panreac], 10 mM magnesium chloride [Merck Chemicals], 1 mM DTT [Calbiochem], 0.2 % Triton X-100 [Merck Chemicals] and protease inhibitor cocktail [Sigma-Aldrich], pH 7.9) or in phosphatase inhibitor buffer (50 mM HEPES [Sigma-Aldrich], 100 mM sodium fluoride [Sigma-Aldrich], 2 mM sodium orthovanadate [Sigma-Aldrich], 2 mM EDTA [Sigma-Aldrich], 1 mM DTT, 0.2 % Triton X-100 and protease inhibitor cocktail). Extracts were disrupted on ice by bath-sonication and vortexed during 45 minutes. Alkaline phosphatase treatment (20 U CIP/2×10^7 tachyzoites, New England Biolabs) was only applied on extracts resuspended in alkaline phosphatase-compatible buffer for 90 minutes at 37º C. Resulting extracts were stored at -80ºC until further analysis.

Tachyzoite extracts were separated by 12.5% SDS-PAGE supplemented with 25 µM Phos-Tag (Wako Pure Chemicals Industries) and 50 µM manganese (II) chloride (Merck Chemicals). After electrophoresis, gels were washed once in 0.1 M EDTA in transfer buffer and transferred onto nitrocellulose membranes. Membranes were incubated with α-rNcNTPase and α-rNcGRA7 PAbs, and then incubated with goat anti-rabbit IgG antibody conjugated to peroxidase. Reactions were developed using 4-chloro-1-naphtol as substrate until signal visualization.

3. Results

*3.1. Molecular characterization of the Nc*NTPase *sequence*

The Nc*NTPase* sequence is currently annotated under the gene ID NCLIV_068400 in the ToxoDB source (chromosome XII, position 6,239,838 to 6,241,718). It is classified as an unspecified product and considered as orthologous gene of Tg*NTPase 1* and *3*. The 1881 bp sequence has no introns and codes for a protein of a predicted molecular weight of ~68.7 kDa (Figure 1A). The predicted protein sequence exhibits a signal peptide of 26 residues, and its EC number is 3.6.1.15 (nucleoside-triphosphate phosphatase). According to the Pfam database, the NcNTPase protein is a member of the GDA1/CD39 (nucleoside phosphatase) family.

Another gene displaying high similarity with the Nc*NTPase* sequence is NCLIV_068460, a predicted gene on chromosome XII, position 6,285,470 to 6,290,398. It codes for an unspecified product which displays 2 introns, with an overall length of 4929 bp and coding for predicted protein of ~109.2 kDa. Our analyses showed that this sequence contains the C-terminus of a potential second Nc*NTPase* copy, and the whole nucleotide sequence of an additional copy (Figure 1A). Hence, and according to ToxoDB, three different *loci* for the Nc*NTPase* gene can be found within the Nc-Liv genome.

BLAST searches for the Nc*NTPase* sequence (NCLIV_068400) in the NCBI database came up with significant alignments for four mRNA annotations, namely XM_003886392.1 (100% identity), AB525222.1 (99% identity), AB010444.1 (99% identity), and XM_003886398.1 (99% identity). The XM_003886392.1, AB525222.1 and AB010444.1 annotations largely corresponded to the Nc*NTPase* gene (NCLIV_068400). However, the AB525222.1 and AB010444.1 sequences differ from the Nc*NTPase* in at least 8 point mutations, and differ from each other in another 10 point mutations (Supplementary file 1). In addition, the XM_003886398.1 sequence corresponded with the NCLIV_068460 predicted gene.

In order to clarify the Nc*NTPase loci* distribution, the Nc-Liv chromosome XII (GenBank: FR823393.1) was analyzed in detail. In contrast to the information available in ToxoDB, three different *loci* containing three tandemly repeated copies of the Nc*NTPase* gene were found. These copies were termed Nc*NTPase 1* (position 6,239,838 to 6,241,718), Nc*NTPase 2* (6,285,037 to 6,286,918) and Nc*NTPase 3* (6,288,517 to 6,290,398) according to their spatial distribution within the genome (Figure 1A). Following this, the XM_003886392.1, AB525222.1, and AB010444.1 annotations were analyzed in the context of the Nc-Liv chromosome XII. The XM_003886392.1 sequence matched perfectly with the Nc*NTPase 1* allele (100% identity), whereas the AB525222.1 and AB010444.1 annotations largely corresponded to the Nc*NTPase 2* and *3* alleles (~99% identity), respectively. However, these two latter sequences showed different point mutations corresponding to the Nc*NTPase 3* and *2* copies, respectively. In addition, and according to our previous observations, the XM_003886398.1 sequence incorporated the last 1449 bp of Nc*NTPase 2*, an intergenic region of 1599 bp, and the full length of the Nc*NTPase 3* copy (Figure 1A).

Our cloning experiments resulted in the isolation of three different Nc*NTPase* alleles, termed clone 1, 2, and 3. These alleles contain between 7 and 8 point mutations, when compared with the original Nc*NTPase* annotation (NCLIV_068400 in ToxoDB, and XM_003886392.1 in GenBank). Interestingly, these clones carry a mixture of point mutations exclusively found in the different Nc*NTPase* alleles within the Nc-Liv genome (Supplementary file 1). Remarkably, the primers employed to amplify the Nc*NTPase* gene anneal to any of the gene duplications, resulting in the unspecific amplification of all the transcribed Nc*NTPase* alleles, regardless of their *loci*. In line with this, six different alleles (three previously published ones and another three isolated by us) coding for the NcNTPase protein were found. In this study, clone no. 3 was expressed in *E. coli* and the resulting recombinant protein was employed to raise polyclonal antibodies in rabbits.

According to the Nc-Liv chromosome XII sequence available in the ToxoDB and GenBank sources (FR823393.1), the Nc*NTPase 2* and *3* copies contain and additional bp (1882 instead of 1881). This is due to the insertion of an additional nucleotide (G, at the position number 8) in a region with tandemly repeated Gs. Strickingly, this insertion alters the reading frame for the mRNA transcription. Unfortunately, the information about this region in the AB525222.1 and AB010444.1 annotations, and clones 1 and 2 is not available due to the cloning process (Supplementary file 1). However, we confirmed the presence of this additional nucleotide by sequencing analyses of the up-stream region on genomic DNA (data not shown).

In order to gain more information on the NcNTPase *loci* distribution within the Nc-Liv genome, the flanking regions of the Nc*NTPase 1, 2* and *3* copies were also analysed in detail by BLAST. As shown in Figure 1B, Nc*NTPase 1* and *3* share almost perfectly identical up- and down-stream sequences. In contrast, these elements were absent in the Nc*NTPase 2* gene. In order to confirm these findings, specific primers were designed to amplify the up-stream sequences of the Nc*NTPase 1*, *2* and *3*. Subsequently, the amplicons were sequenced and analyzed in detail (Supplementary file 2). Fragments comprising 1331, 536 and 1285 bp were obtained in two directions from the Nc*NTPase 1*, *2* and *3* up-stream regions, respectively. All the sequences were identical to the originally published ones, and confirmed the upper distribution of the Nc*NTPase 1*, *2* and *3* copies.

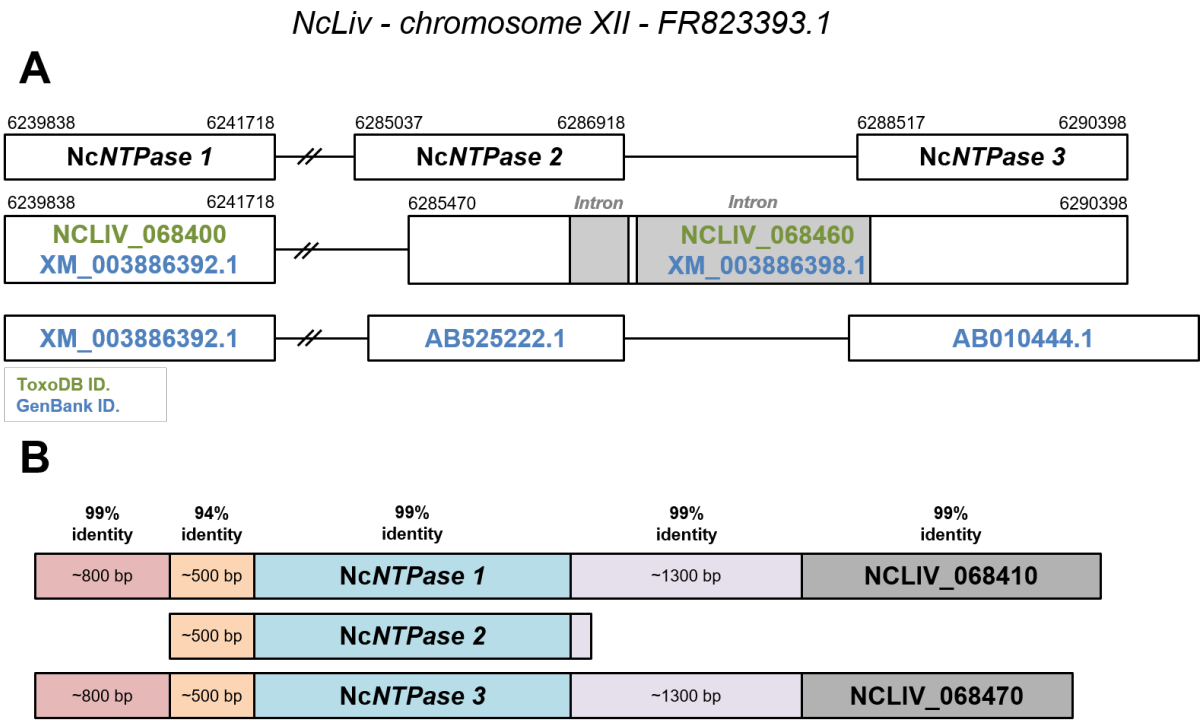

Figure 1: A: Schematic representation of the Nc*NTPase loci* in the Nc-Liv genome in comparison with the available sequences at the ToxoDB and GenBank sources. Accesion numbers, genomic positions and introns are represented on the graphic. B: Schematic representation of the Nc*NTPase 1*, *2* and *3* gene structure, according to BLAST analyses.

3.2. Characterization of NcNTPase protein

The identity of the recombinant NcNTPase (gene ID: NCLIV_068400) was corroborated by mass spectrometry (theorethical molecular weight: 69,9 kDa, Score: 214; mass values matched: 25/65; sequence coverage: 47%). Hence, the recombinant protein was employed to develop specific polyclonal antibodies (PAbs).

Immunoblotting of Nc-Liv extracts separated by 1D-SDS-PAGE under reducing conditions revealed that the polyclonal α-rNcNTPase antiserum reacted with a main band of approximately 64 kDa (Figure 2A). On immunoblots of Nc-Liv extracts separated by 2D-electrophoresis, the polyclonal α-rNcNTPase antiserum recognized a chain of 5 spots with an approximate *Mr* of 60-70 kDa but different isoelectric points, which were located in the acidic range of the pH gradient (Figure 2B). The same affinity purified antiserum was employed for immunogold TEM, which showed that the NcNTPase protein is clearly associated with the tachyzoites dense granules (Figure 2C).

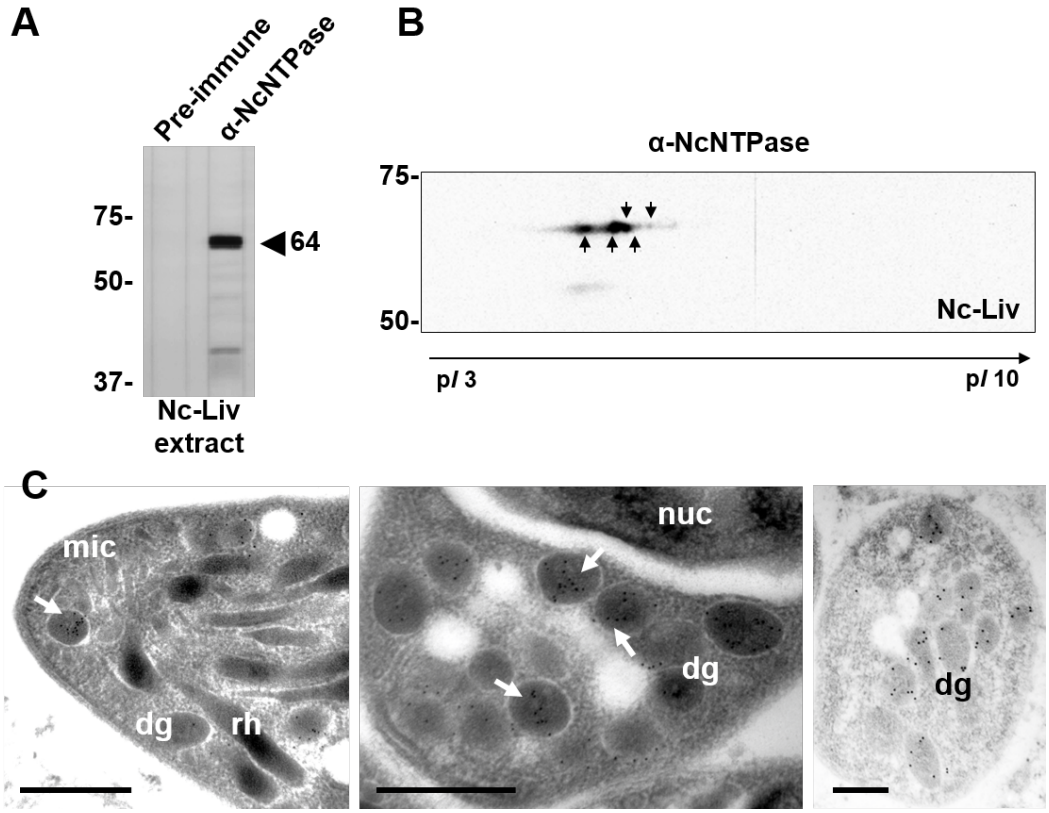

Figure 2: A: *N. caninum*-based Western-blot showing the immuno-reactivity of α-rNcNTPase antibodies against parasite extracts. A main band of approximately 64 kDa was detected. B: NcNTPase-immunome profile of the tachyzoite stage of the Nc-Liv isolate. Proteins were separated along a non-linear pH gradient (pH 3-10 NL, IPG strips; 17 cm) in the first dimension and on a 10% polyacrylamide gel in the second dimension. Following transfer to PVDF membranes, a polyclonal rabbit antiserum raised against rNcNTPase was used. The 2-DE immunoblot was analyzed with the PD-Quest software. Arrows indicate the detected protein spots. C: Immunogold TEM of *N. caninum* tachyzoites labelled with the α-rNcNTPase antibody. The images show in detail the apical region and the dense granules in longitudinal and cross sections. The NcNTPase is clearly associated with dense granules (arrow). Dense granules (dg), micronemes (mic), rhoptries (rh) and the nucleus (nuc) are indicated on the micropgraphs. Bars represent 0.5 µm.

*3.3. Comparative analysis of Nc*GRA7 *and Nc*NTPase *mRNA expression levels during the lytic cycle*

Nc*NTPase* and Nc*GRA7* transcripts were quantified at different timepoints, namely shortly after invasion (6 hpi), PV maturation (24 hpi), exponential growth of parasites (48 hpi) and tachyzoite egress (56 hpi), using Nc*TUBα* as normalizer gene. Both dense granule genes displayed a similar mRNA transcription pattern during the lytic cycle (Figure 3A), showing the lowest mRNA levels at 24 hpi and the highest at 6 and 56 hpi (P<0.005; Kruskal-Wallis test and Dunn's test). Significant differences in mRNA levels were also observed at 6 and 48 hpi for both proteins (P<0.005; Kruskal-Wallis test and Dunn's test). Consequently, mRNA levels at 24 hpi were used as baseline to calculate transcript

fold increases. Nc*GRA7* displayed a 3-fold increase in mRNA levels at 6 and 56 hpi. Similarly, Nc*NTPase* showed a 5-fold increase at 6 hpi and a 4-fold increase at 56 hpi. Both genes displayed a 2 fold increase at 48 hpi. Results were similar when Nc*SAG1* was used as a normalizer gene (data not shown).

Nc*NTPase* and Nc*GRA7* mRNA levels were also assessed after DTT stimulation, which leads to tachyzoite egress. Upon DTT treatment, tachyzoites exhibited significant increases in mRNA levels for both genes (P<0.005; Kruskal-Wallis test) (Figure 3B). Similarly to naturally occurring egress, Nc*GRA7* mRNA levels were 3-fold increased, while those coding for Nc*NTPase* displayed a 6-fold increase.

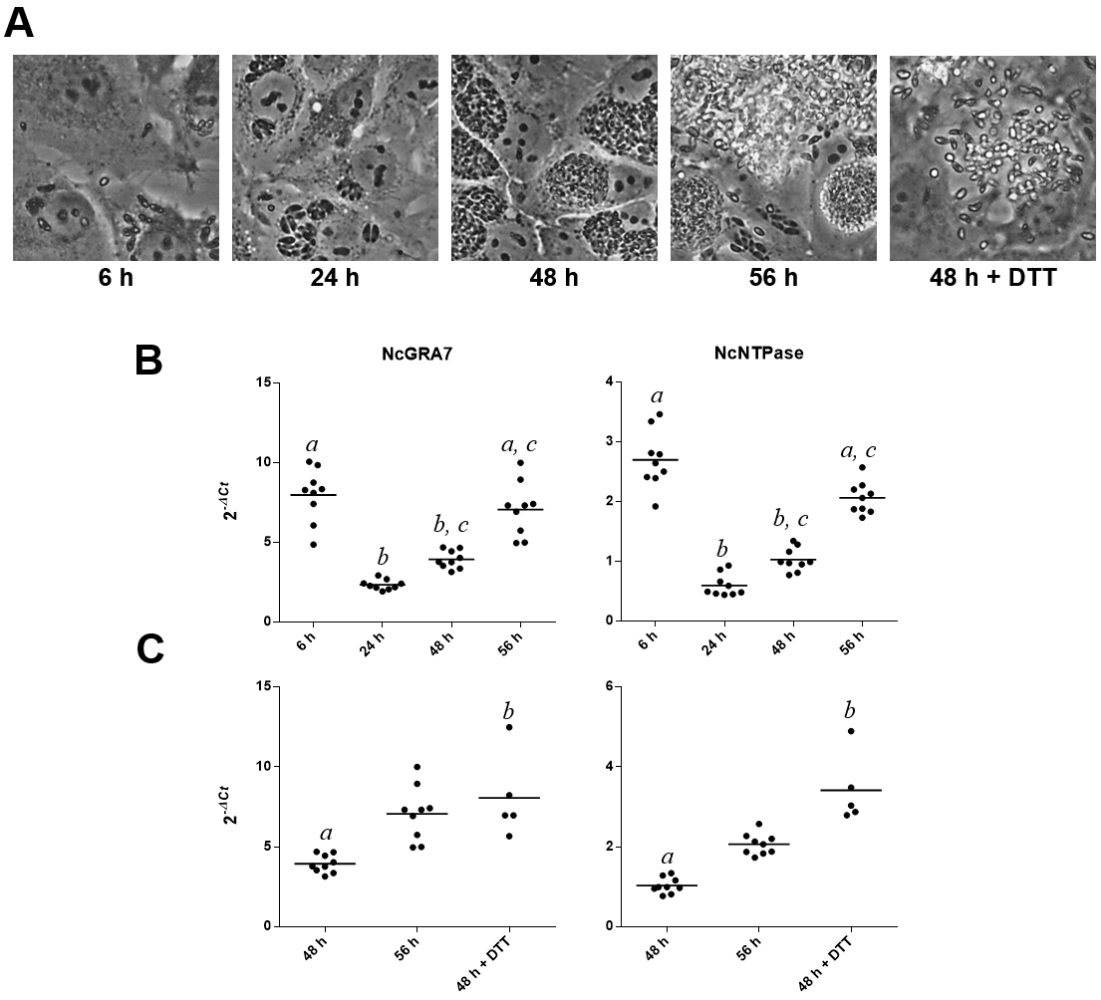

Figure 3: mRNA expression of Nc*GRA7* and Nc*NTPase*. Real time-PCR was employed to assess the mRNA expression of both proteins along the lytic cycle. A: photomicrographs showing the infection dynamics of the Nc-Liv isolate on MARC-145 cultures at recent invasion (6 h), PV maturation (24 h), exponential growth of parasites (48 h) and tachyzoite egress (56 h and 48 h + DTT). B: mRNA expression levels of Nc*GRA7* and Nc*NTPase* during the lytic cycle. C: Effect of DTT supplementation to artificially induce egress at 48 h on mRNA expression for both proteins. For A and B, each point represents a single sample and bars represent the mean value. *a*, *b* and *c* indicate significant differences (p<0.005; Kruskal-Wallis test).

3.4. Immunolocalization dynamics of NcGRA7 and NcNTPase throughout the lytic cycle

Herein, three different fixatives were employed in order to obtain a concise picture of the localization of these two dense granule proteins throughout the lytic cycle. In general, methanol fixation retained more efficiently the reservoirs of intracellular NcNTPase and NcGRA7, whilst paraformaldehyde and glutaraldehyde fixation further preserved the protein after secretion.

Immunofluorescence staining using anti-NcNTPase antibodies confirmed that NcNTPase is distributed throughout the tachyzoites cytoplasm exhibiting a punctate staining pattern that is reminiscent for dense granules (Figure 4). NcNTPase secretion was clearly observed in most of the analyzed time points, and could be detected during early invasion, PV maturation, and egress. In addition, the NcNTPase localized to the PVM as soon as the PV was formed. This was particularly visible in samples fixed in the presence of paraformaldehyde and glutaraldehyde (Figure 4). Compared to NcNTPase, the recognition pattern of anti-NcGRA7 antibodies observed by immunofluorescence was less distinctive. Protein secretion was also evident during the early invasion, PV maturation and egress, and PVM association was observed (Figure 5A).

On the other hand, co-immunolocalization studies revealed differences in the distribution pattern of the NcNTPase and NcGRA7 proteins during invasion (1 h) and egress (56 h). At these time points, NcNTPase was scattered throughout the entire tachyzoite cytoplasm, whereas NcGRA7 labeling was restricted to more specific areas close to the parasite surface. By contrast, during PV maturation (24 h), NcNTPase and NcGRA7 colocalized much more closely (Figure 5B).

3.5. Differential effects of calcium on NcNTPase and NcGRA7 secretion

Freshly purified tachyzoites were incubated in the presence of A23187 and ethanol at 37 ºC to induce calcium-mediated secretion of dense granule components. In addition, the effects of DTT supplementation on protein secretion were also investigated at 37 ºC. Immunoblot analyses revealed that NcNTPase and NcGRA7 were present in all secreted fractions of both treated and non-treated parasites (Figure 6). However, NcGRA7 secretion was noticeably increased after A23187, ethanol and DTT treatment, and the pronounced NcNTPase release was not apparently influenced by any chemical supplementation or temperature increase (Figure 6). Calcium-mediated protein secretion was validated by NcMIC2 secretion upon A23187, ethanol or DTT treatment, and tachyzoite lysis was discarded by the lack of detection of NcTUBα in all culture supernatant fractions (Figure 6).

3.6. Phosphorylation of NcNTPase and NcGRA7

Phosphorylation of NcNTPase and NcGRA7 was studied at 56 hpi to determine whether this post-translational modification is involved in protein regulation. For this purpose, tachyzoite extracts were processed under conditions that preserve the phosphorylation status of each protein and resolved by Phos-Tag SDS-PAGE electrophoresis. Both NcNTPase and NcGRA7 showed an electrophoretic

mobility shift in those extracts treated with phosphatase inhibitors, which suggests that both proteins were phosphorylated at 56 hpi (Figure 7).

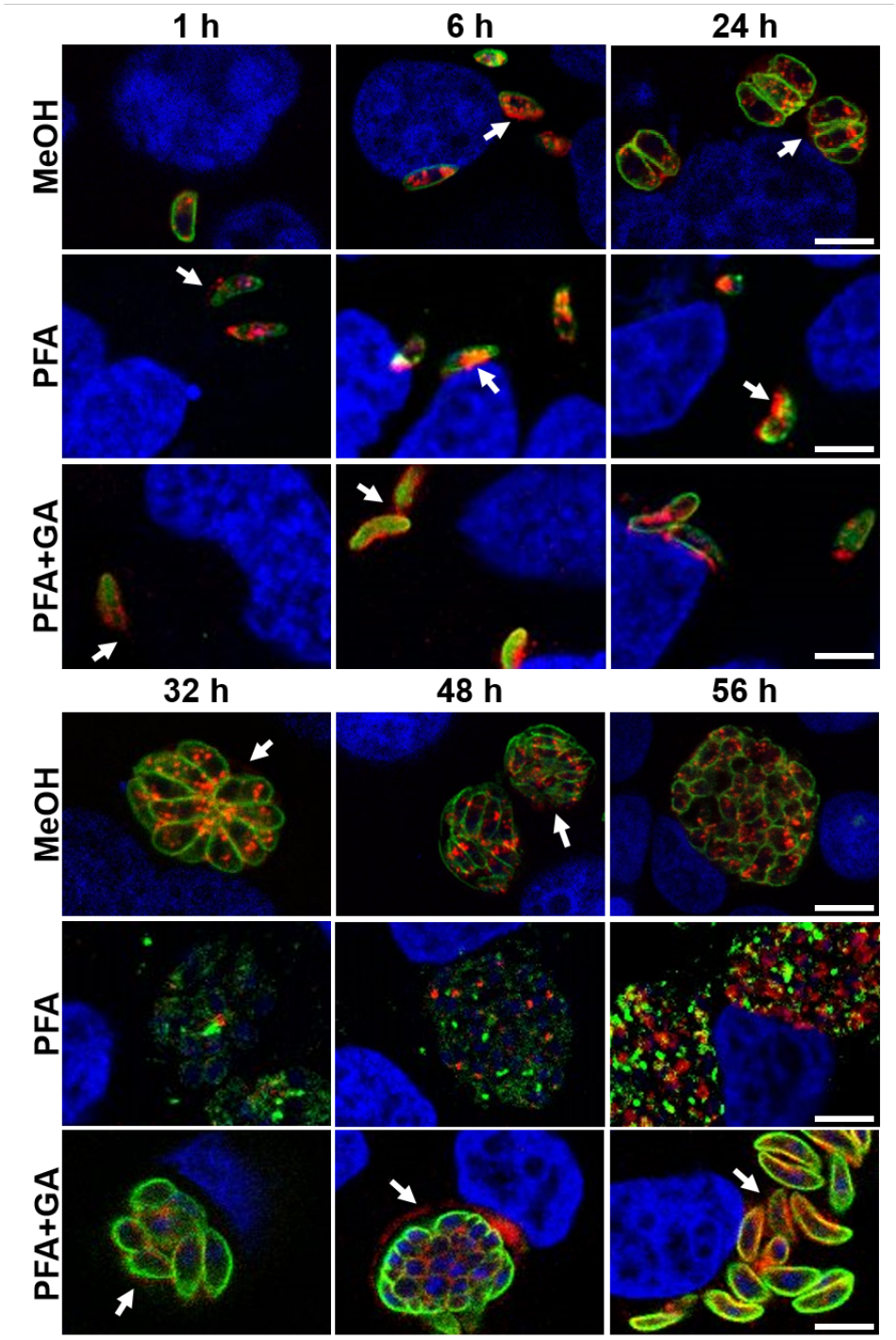

Figure 4: Confocal laser scanning microscopy of NcNTPase along the lytic cycle of tachyzoites. Infected cultures were fixed with methanol (MeOH), paraformaldehyde (PFA) and paraformaldehyde combined with glutaraldehyde (PFA+GA). Then, coverslips were double labelled with affinity purified antibodies against NcNTPase (red) and monoclonal antibodies against NcSAG1 (green). Nuclei were stained with DAPI (blue). All the images show a single 1 µm slice. Bars represent 4 µm.

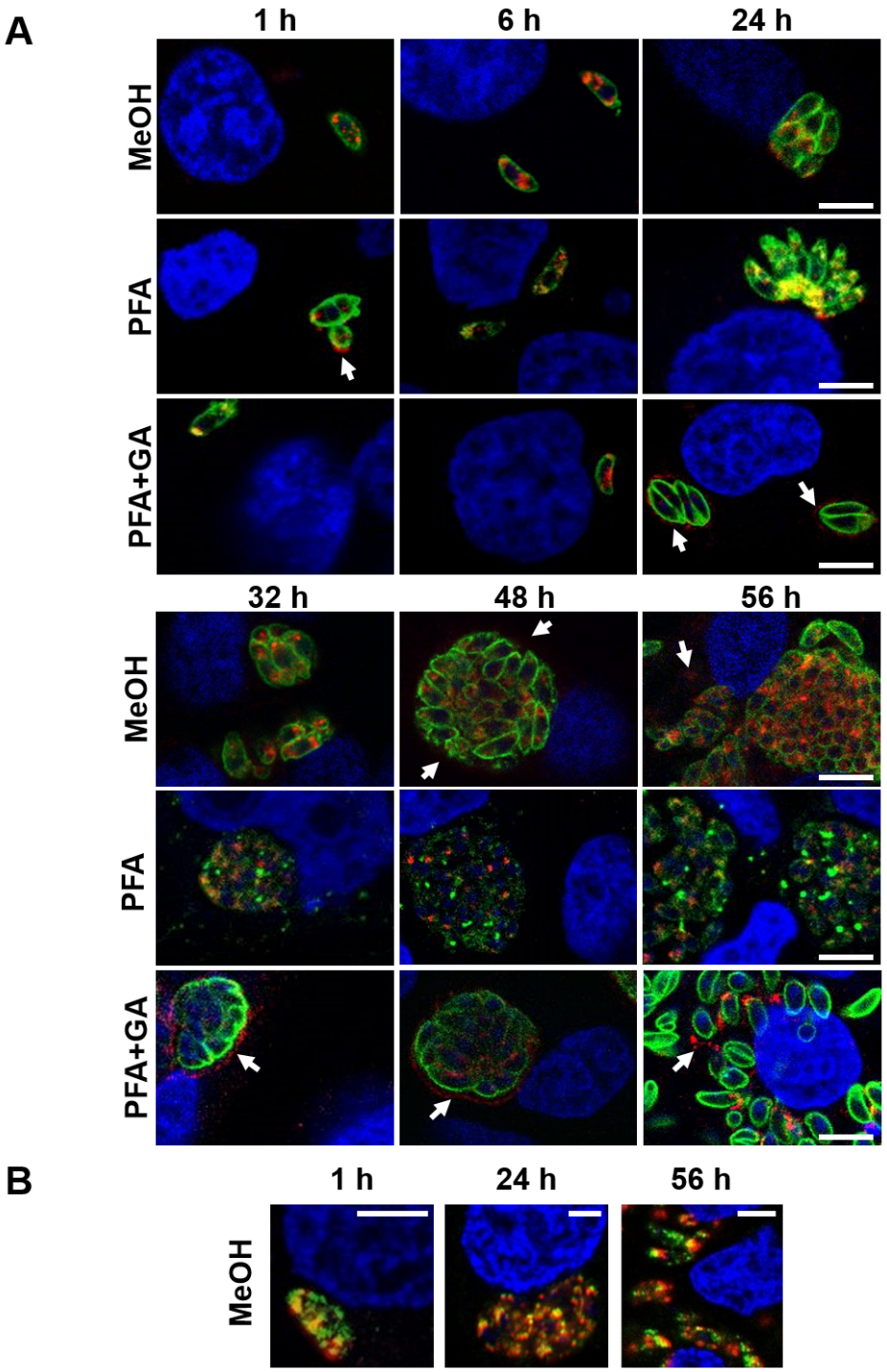

Figure 5: A: Confocal laser scanning microscopy of NcGRA7 along the lytic cycle of tachyzoites. Infected cultures were fixed with methanol (MeOH), paraformaldehyde (PFA) and paraformaldehyde combined with glutaraldehyde (PFA+GA). Then, coverslips were double labelled with affinity purified antibodies against NcGRA7 (red) and monoclonal antibodies against NcSAG1 (green). Nuclei were stained with DAPI (blue). B: Confocal laser scanning microscopy of NcNTPase (green) and NcGRA7 (red) along the lytic cycle of tachyzoites. Methanol-fixed cultures were double labelled with affinity purified antibodies against NcNTPase (green) and monoclonal antibodies against NcGRA7 (red). Nuclei were also stained with DAPI (blue). All the images show a single 1 µm slice. Bars represent 4 µm.

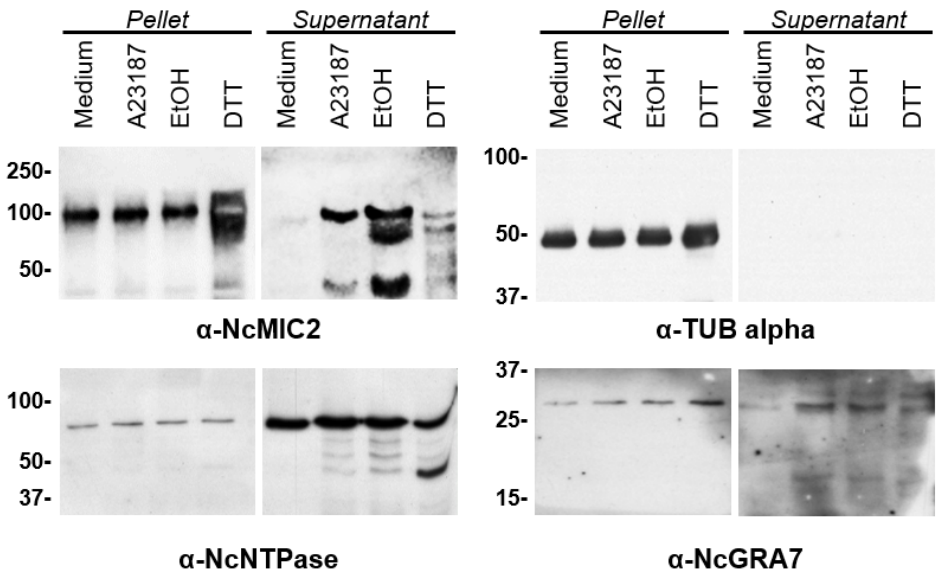

Figure 6: Effect of A23187, ethanol and DTT on secretion of NcNTPase and NcGRA7 proteins as shown by Western-blot using respective antibodies. The same protein samples were also probed by immunoblot analyses with α-NcMIC2 and α-TUBα antibodies to confirm induced secretion and exclude inadvertent tachyzoite lysis, respectively. NcNTPase and NcGRA7 discharge was observed in culture supernatants regardless of the treatment. In contrast, NcMIC2 secretion was only evident after A23187, ethanol and DTT supplementation. Tachyzoite lysis was not detected. All the antibodies specifically reacted against their respective protein on tachyzoite extracts.

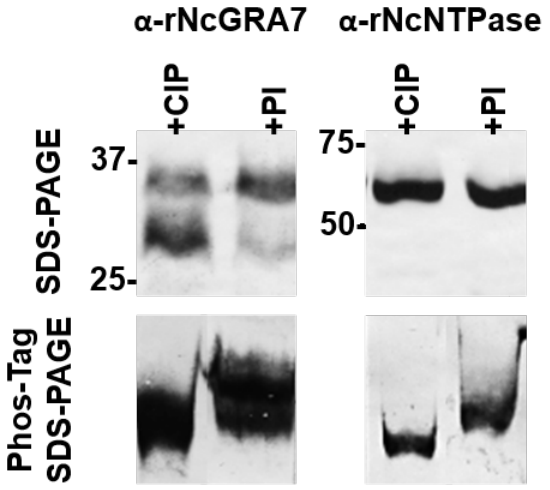

Figure 7: Phosphorylation detection of NcGRA7 and NcNTPase by Phos-Tag SDS-PAGE. Tachyzoite extracts obtained at 56 hpi were processed with alkaline phosphatase (CIP) and phosphatase inhibitors (PI), electrophoresed on SDS-PAGE and Phos-Tag SDS-PAGE gels, and blotted to nitrocellulose membranes. Then, both proteins were detected by their respective antibodies in order to detect a mobility shift of the proteins treated with PI.

4. Discussion

In the present work we performed for the first time a comprehensive study on the localization dynamics, secretion, phosphorylation, and mRNA expression profiles of the two dense granule proteins NcNTPase and NcGRA7 throughout the tachyzoites lytic cycle. Although the previously characterized NcGRA7 was included in this study mainly for comparative purposes, we present some new and undescribed aspects of this GRA protein. Moreover, we propose a new model for the NcNTPase genomic distribution supported by different approaches.

In *N. caninum* the NcNTPase protein has been shown to be more abundant in virulent isolates (Regidor-Cerrillo *et al.*, 2012). However, several functional aspects of this protein still need to be elucidated. Overall, NTPases (also named as NTPDases in the literature) are found in almost all eukaryotes. These enzymes present apyrase conserved regions to hydrolyze nucleoside triphosphates and diphosphates in the presence of divalent cations (Sansom, 2012). The NTPases are also present in both *T. gondii* and *Sarcocystis neurona* (Zhang *et al.*, 2006; Asai *et al.*, 1998; Asai *et al.*, 1995). Intriguingly, orthologues of NTPase are not found in *Eimeria* and *Plasmodium* parasites, indicating that this protein may participate in some conserved processes among the Sarcocystidae family members (Zhang *et al.*, 2006; Asai *et al.*, 1998). In *T. gondii,* some NTPase isoforms are restricted to the virulent strains (Asai *et al.*, 1995). Nevertheless, there is no consensus regarding their specific function, although it has been proposed they participate in purine salvage to supply energy (Sibley *et al.*, 1994), to be involved in tachyzoitye intracellular proliferation and egress (Nakaar *et al.*, 1999; Silverman *et al.*, 1998), and in the suppression of the host immune responses by interfering in purinergic signaling (Tonin *et al.*, 2013; Sansom, 2012; Melo *et al.*, 2011; Santos *et al.*, 2009).

Herein, we describe the presence of three different *loci* for the Nc*NTPase* gene within the Nc-Liv genome. Our sequence analyses showed that Nc*NTPase 2* lacks the up and down-stream elements shared by Nc*NTPase 1* and *3*. Interestingly, *T. gondii* only expresses the Tg*NTPase 1* and *3*, which is under the control of an active promoter solely present in those alleles (Nakaar *et al.*, 1998; Asai *et al.*, 1995). This is indicative of a similar promotor structure for the Nc*NTPase* alleles. In addition, we identified up to six different Nc*NTPase* alleles. Previous studies in *T. gondii* reported on the presence of three tandemly repeated copies of the Tg*NTPase* gene (Bermudes *et al.*, 1994). Asai and colleagues then also detected up to three different alleles of the Nc*NTPase* gene by Southern-blot analyses in *N. caninum* (Asai *et al.*, 1998). In the present work we also demonstrate the presence of diverse protein species of the NcNTPase in the Nc-Liv isolate, which is in accordance with previous studies (Regidor-Cerrillo *et al.*, 2012; Shin *et al.*, 2005b; Lee *et al.*, 2003). Therefore, it is likely that *N. caninum* expresses different isoforms of the NcNTPase protein as reported earlier for *T. gondii* (Kikuchi *et al.*, 2001). According to this, further studies should be carried out in order to clarify the impact of the NcNTPase expression on parasite pathogenicity. However, the Nc*NTPase* 2 and 3 copies show a reading frame shift caused by a single-nucleotide insertion, and thus, it is unlikely that both genes are properly translated.

Remarkably, it has been recently described that gene duplications are notoriously underestimated due to collapsing of the assembly in regions containing tandemly duplicated clusters of similar genes (Adomako-Ankomah *et al.*, 2014). More specifically, Adomako-Ankomah and colleagues have evidenced the existence of expanded *loci* carrying a variable number of alleles coding for the TgNTPase protein, the numbers of which depend on the isolate. These findings support our evidence on the wide variety of transcribed Nc*NTPase* alleles that we have observed, which could be harboured within the Nc*NTPase loci*. Consistent with this, we failed to amplify the whole Nc*NTPase 1* gene (coding sequence and flaking regions) by PCR, even when employing specific long-range PCR methods (data not shown). In this scenario, the availability of different genome annotations from diverse *N. caninum* isolates would be highly desirable, but to date only the Nc-Liv genome has been fully sequenced (Reid *et al.*, 2012).

By immunogold TEM, the NcNTPase was localized to the dense granules of intracellular tachyzoites employing a specific anti-NcNTPase antibody. These findings are in accordance with a previous study in which a similar pattern was observed when extracellular tachyzoites were labelled with an α-TgNTPase antibody (Asai *et al.*, 1998). In addition, we performed a detailed immunofluorescent tracing of the NcNTPase and NcGRA7 proteins throughout the lytic cycle by using three different fixation protocols. NcNTPase and NcGRA7 secretion was easily detected during early invasion, PV maturation, and egress no matter which fixation was employed. Besides, both proteins associated with the vacuole periphery, indicating that they might interact with the PVM, as previously reported for TgNTPase (Bermudes *et al.*, 1994) and the NcGRA7 proteins (Aguado-Martínez *et al.*, 2010). However, this particular localization was more evident after combined paraformaldehyde-glutaraldehyde fixation, and almost inappreciable after methanol and paraformaldehyde fixation. Moreover, as shown by the co-localisation studies, the temporal distribution of the NcNTPase and NcGRA7 proteins were distinct during invasion and egress, suggestive of differential protein trafficking at these stages of the lytic cycle. However, NcNTPase is clearly a canonical GRA protein according to earlier described criteria (Mercier & Cesbron-Delauw, 2015): it has a relatively low molecular weight, the protein carries a N-terminal signal peptide, it colocalized with NcGRA7 within the dense granules, was secreted into the tachyzoite PV, and, similar to NcGRA7, remained there until parasites underwent egress.

The expression of Nc*NTPase* and Nc*GRA7* transcripts was quantified in infected MARC-145 throughout the lytic cycle. For both, the highest mRNA levels were detected at 6 and 56 hpi, coinciding with egress and/or early invasion. Therefore, NcNTPase and NcGRA7 function may be necessary to guarantee the lytic cycle progression. A similar expression pattern was described previously for the rhoptry proteins NcROP40 and NcROP2Fam-1 (Pastor-Fernández *et al.*, 2015b), highlighting the relevance of rhoptry and dense granules proteins for the invasion process in *N. caninum*. This is in accordance with previous studies carried out with *Plasmodium falciparum* and *T. gondii* that

showed that the changes in mRNA levels at specific points of the lytic cycle have a strong influence on the parasite's developmental transitions (Radke *et al.*, 2005; Le Roch *et al.*, 2004). In line with this, a modal switch from expression of proteins involved in invasion and motility of *T. gondii* has been also described in egressed tachyzoites (Gaji *et al.*, 2011; Lescault *et al.*, 2010).

On the other hand, we quantified the Nc*NTPase* and Nc*GRA7* mRNA levels after inducing *N. caninum* egress *in vitro* by DTT treatment (Pastor-Fernández *et al.*, 2015b; Esposito *et al.*, 2007). Significant increases in their mRNA levels were observed after DTT supplementation, suggesting that both proteins may be relevant during the egress or subsequent phases. Similar findings were observed for the NcROP2Fam-1 protein, whilst Nc*ROP40* mRNA expression was not affected by DTT supplementation (Pastor-Fernández *et al.*, 2015b). Interestingly, *T. gondii* secretes the reducing agent glutaredoxin to activate the TgNTPase as replication increases, supporting our hypothesis regarding the crucial role of the protein function during egress. Indeed, DTT has been shown to activate the TgNTPase protein *in vitro* and to trigger egress of tachyzoites (Krug *et al.*, 2013; Stommel *et al.*, 2001). Although the processes governing egress are not fully understood, intracellular calcium levels appear to trigger the abrupt exit of parasites from the PV, which is accompanied by a rapid decrease in host cell ATP mediated by TgNTPase activation (Blackman & Carruthers, 2013). Hence, we hypothesize that egress regulation in *N. caninum* relies on NcNTPase activation, which is accompanied by an increase in the expression of secreted effectors (NcGRA7 and NcROP2Fam-1) necessary to accomplish new invasion waves in the neighbouring cells (Pastor-Fernández *et al.*, 2015b). In fact, TgNTPase has been previously suggested to act as a timer for the *T. gondii* lytic cycle (Santos *et al.*, 2009). Since egress and invasion represent critical processes in which parasites are highly exposed to the host immune system, NcNTPase secretion could counteract the development of inflammatory responses to avoid tachyzoite clearance. Interestingly, it has been suggested that the TgNTPase may suppress the local host immune responses (Tonin *et al.*, 2013; Sansom, 2012; Melo *et al.*, 2011; Santos *et al.*, 2009), supporting this hypothesis.

Herein, we found that NcNTPase secretion occurs in tachyzoites undergoing egress regardless of the applied treatment (A23187, ethanol, or DTT). In addition, a significant NcNTPase secretion was also observed on parasites kept in ice. Similar findings have been previously reported for the SnNTPase protein from *S. neurona*, which can be detected on secreted fractions after incubation at 37 ºC without further treatment (Zhang *et al.*, 2006). The NcGRA7 protein displayed a similar behaviour, although secretion at 4º C was apparently less pronounced. According to this, it has been showed that *T. gondii* GRA proteins are constitutively released in a calcium-independent and a temperature-dependent fashion (Phelps *et al.*, 2008; Carruthers, 2002; Chaturvedi *et al.*, 1999). However, our results suggest that NcNTPase discharge is under a distinct regulation mechanism than the NcGRA7 protein. In contrast to dense granules, microneme secretion can be specifically induced by calcium

ionophores and DTT both in *T. gondii* and *N. caninum* (Pastor-Fernández *et al.*, 2015b; Naguleswaran *et al.*, 2001; Carruthers & Sibley, 1999). According to this, all the applied treatments specifically induced NcMIC2 secretion as described earlier (Pastor-Fernández *et al.*, 2015b; Lovett *et al.*, 2000), whereas they had no effect on NcNTPase secretion.

Intriguingly, both NcNTPase and NcGRA7 are phosphoproteins. This protein modification may indicate a common regulation mechanism necessary for their participation within the lytic cycle. We have recently employed the same Phos-Tag SDS-PAGE approach to determine the phosphorylation state of the two rhoptry proteins NcROP40 and NcROP2Fam-1, and found that NcROP2Fam-1 protein was also phosphorylated (Pastor-Fernández *et al.*, 2015b). Previous studies have also demonstrated the phosphorylation of TgROP2, TgROP4 and TgGRA7, but only in intracellular parasites (Dunn *et al.*, 2008; Carey *et al.*, 2004; Neudeck *et al.*, 2002). Interestingly, phosphorylation of TgGRA6 has been shown to coincide with its association with the PVM (Mercier *et al.*, 2005; Labruyere *et al.*, 1999), and this could also be the case for NcNTPase and NcGRA7 proteins, which are also localized at the periphery of the PV. Nevertheless, TgGRA7 was shown to associate with the PVM independently of its phosphorylation status (Dunn *et al.*, 2008; Coppens *et al.*, 2006; Neudeck *et al.*, 2002). In fact, it was suggested that TgGRA7 phosphorylation might regulate the formation of complexes with other GRA proteins to facilitate secretion of transmembrane-domain containing proteins (Braun *et al.*, 2008).

In conclusion, we have unraveled, at least partially, the complex NcNTPase genome organization, and demonstrate that three different *loci* and up to six different alleles of the Nc*NTPase* gene exist. However, the improvement of the current gene annotation within the Nc-Liv genome and the incorporation of new fully sequenced isolates would be highly desirable in order to clarify more accurately the distribution of the Nc*NTPase* genes. The present work aimed to characterize the NcNTPase and NcGRA7 proteins through an integrative and descriptive approach in the context of the host-parasite relationship. NcNTPase and NcGRA7 are up-regulated, secreted and phosphorylated during egress and early invasion, which suggests that both are likely involved in these and the subsequent phases of the lytic cycle. Nevertheless, the specific role of the NcNTPase and NcGRA7 proteins remains to be elucidated. In this sense, reverse genetics would be useful to determine the NcNTPase and NcGRA7 function, and to assess the impact of NcNTPase expression in the pathogenicity displayed among isolates.

Author contributions

JRC, GAG and LMOM conceived and designed the experiments. IPF, PGL, VMH and AH performed the experiments. IPF, JRC, GAG and LMOM analyzed the data. IPF, JRC, GAG, PGL, VMH, AH and LMOM wrote the paper.

Acknowledgements

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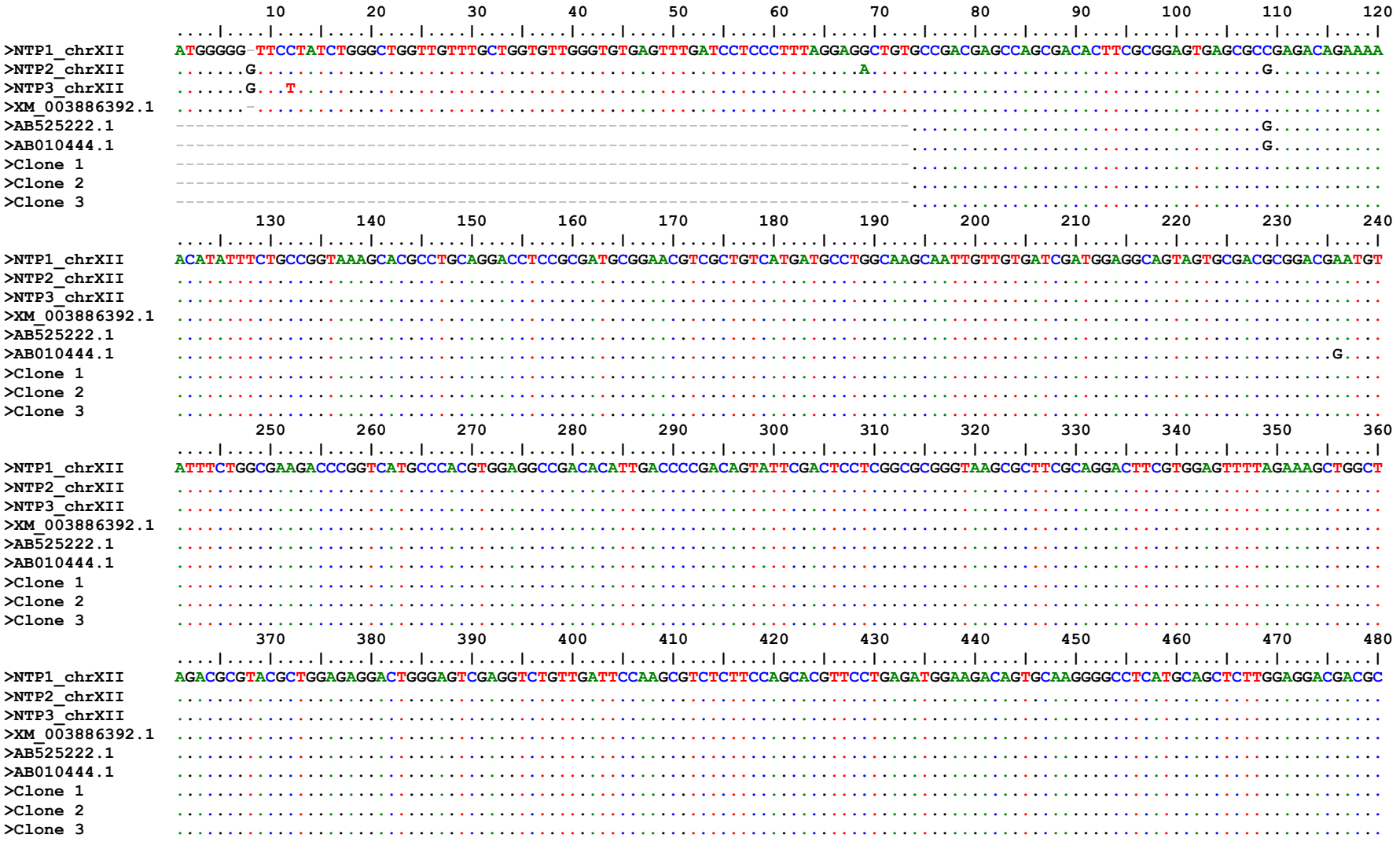
Supplementary figure 1: sequence alignment of the Nc*NTPase* copies and clones



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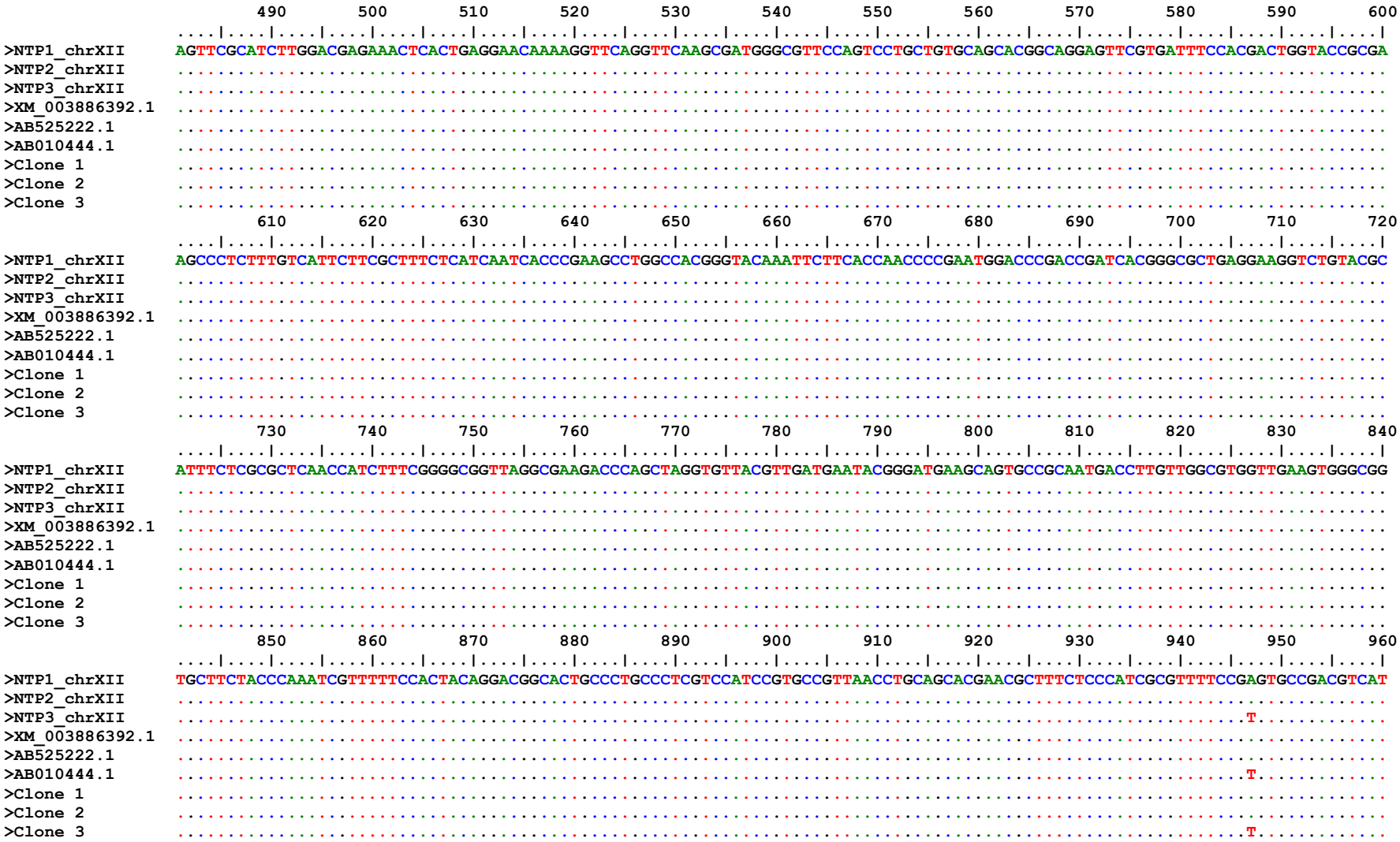

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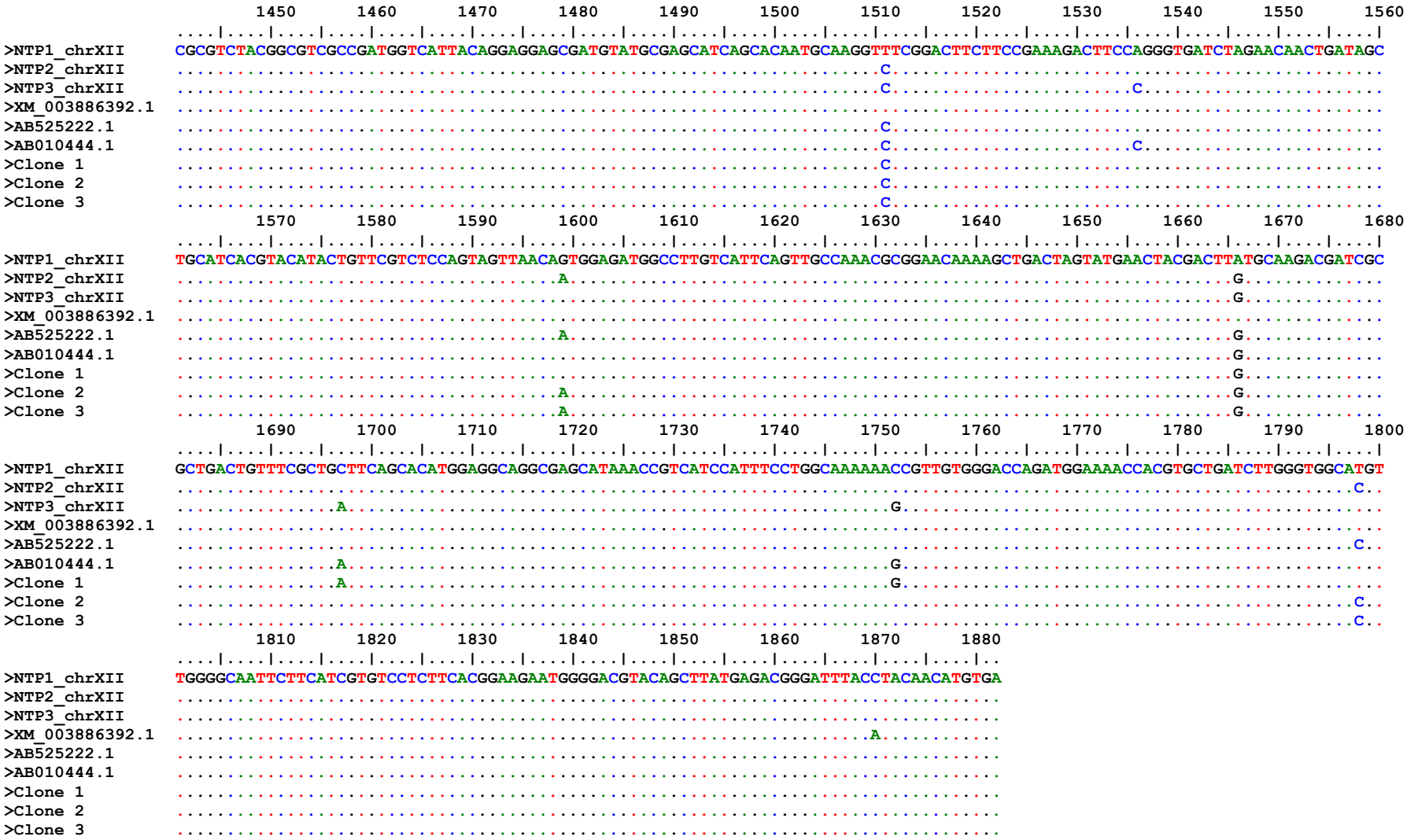
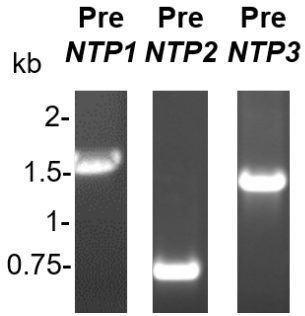
                970       980       990       1000      1010      1020      1030      1040      1050      1060      1070      1080
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
>NTP1_chrXII    ATCGGTTTCTTTTATGCAGCTGGGTGTGGCCAGTTCCTCCGGACTGTTTTTCAAGGAGCTGTGCAGTAACGCCGAATTTCGGCATCAGGGGATTTGCTATAATCCCTGTATTTTCAGAGG
>NTP2_chrXII    ................................................................................................................................
>NTP3_chrXII    ................................................................................................................................
>XM_003886392.1 ................................................................................................................................
>AB525222.1     ................................................................................................................................
>AB010444.1     ................................................................................................................................
>Clone 1        ................................................................................................................................
>Clone 2        ................................................................................................................................
>Clone 3        ................................................................................................................................
                1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
>NTP1_chrXII    CTTCCGACAGGCCTGTTCCGCGGGCGATGTCGAGATCCTTCCAGACGGAACCATAGTTGTGGATGAAGATGTGCGAAAGAACAAGCTGAAGCCGGTGGCGACATCTTGCTCAGCGAACAA
>NTP2_chrXII    ...........................................................................................A....................
>NTP3_chrXII    ................................................................................................................................
>XM_003886392.1 ................................................................................................................................
>AB525222.1     ...........................................................................................A....................
>AB010444.1     ................................................................................................................................
>Clone 1        ...........................................................................................A....................
>Clone 2        ...........................................................................................A....................
>Clone 3        ...........................................................................................A....................
                1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
>NTP1_chrXII    TCCGGAAATTAGTTTCAAGGCAATGAATGAAATGCAATGTCGCGAAAACAAGATTGATCCGACGAAATCACTCGCGGAGCGGCTGAGGATCGACGACTGCTTCCAGATTGTAGGAACTGG
>NTP2_chrXII    ..............................T..........T.....................................................................
>NTP3_chrXII    ................................................................................................................................
>XM_003886392.1 ................................................................................................................................
>AB525222.1     ..............................T..........T.....................................................................
>AB010444.1     ................................................................................................................................
>Clone 1        ..............................T..........T.....................................................................
>Clone 2        ..............................T..........T.....................................................................
>Clone 3        ..............................T..........T.....................................................................
                1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
>NTP1_chrXII    TGATTTCGACACATGCCAAGCCCAGGTAGAAGAACTTCTCGTCAGTCCCAGGTTTCCACTTCCAGCGAACATTGAGGCAGCGTCGTCAGGCTTCGAATCTGTTGGTCAGGTTTTCAAGTT
>NTP2_chrXII    ................................................................................................................................
>NTP3_chrXII    ................................................................................................................................
>XM_003886392.1 ................................................................................................................................
>AB525222.1     ................................................................................................................................
>AB010444.1     ................................................................................................................................
>Clone 1        ................................................................................................................................
>Clone 2        ................................................................................................................................
>Clone 3        ................................................................................................................................
```

Supplementary figure 1 (continuation):



Supplementary figure 2: Sequence alignment of the up-stream regions of the Nc*NTPase 1*, *2* and *3*. Specific primers were designed to amplify the up-stream sequences of the Nc*NTPase 1*, *2* and *3* as indicated in the table below. Forward primers were specific for the Nc*NTPase 1*, *2* and *3* sequences, whilst reverse primer was common for all the copies. PCR amplification yielded a single fragment with the expected molecular weight (see figure below).

Nc*NTPase* copy	Forward primer	Reverse primer	Expected amplicon length
1	CCTGCTCCATAAAGAACGTACCTA	CAATTGTTGTGATCGATGGAGG	1599 bp
2	CGAAAACGCGGTGTAGAGG		752 bp
3	GTCTCATAAATTTTGCAACCAGCGA		1544 bp




```
                    10        20        30        40        50        60        70        80        90        100       110       120       130
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1            ATGACTACCGACACCAAGTTCCTTATGATTGCTGTACAAATACAACCAGCTATAGATTAAAGAATCTACACGCGAGTGTAACGAAAAAACTGGAGCCTATTTCCCAGCGGAGAAGCGCAATCTTTTGCCG
Pre-NTP2            ----------------------------------------------------------------------------------------------------------------------------
Pre-NTP3            ----------------------------------------------------------------------------------------------------..........................

                    140       150       160       170       180       190       200       210       220       230       240       250       260
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1            TGCCACACACAGTCAGCAAGGTGAGGGACTGTGCCCGTGCAGCCCATCCTACTCTATACCAGAAACAGGTACTGCGAGCGACACTGTGCGAAATTGTGTTTGTGTTTTGGGGGAAAAAACTTAAGACTAG
Pre-NTP2            ----------------------------------------------------------------------------------------------------------------------------
Pre-NTP3            ..........................................................................................................................

                    270       280       290       300       310       320       330       340       350       360       370       380       390
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1            CTAACGCTTCACGTCGCAGCACCGCCTCTGTAGATCATCCACAAACAGATCCTCTACACATCGCTTCCCCGACCCAGCCTCGACAATCTACAAGCAACCGCACTGTGCGGCAGCCGTGACGTGCTCACTG
Pre-NTP2            ----------------------------------------------------------------------------------------------------------------------------
Pre-NTP3            ..............................G.....................G.....................................................................

                    400       410       420       430       440       450       460       470       480       490       500       510       520
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1            AGTCACTTCACAAACTCCGGACGATAGACGCAACCCCCTCTTATACTAAAGGATCATGGTCATCTTATTACCTTTCTTCAATGACGACTTCCCTCCATTCCCCGACCCACCGCACTCCGATATCCTCGAT
Pre-NTP2            ----------------------------------------------------------------------------------------------------------------------------
Pre-NTP3            ..G.....................................................................................................................G.

                    530       540       550       560       570       580       590       600       610       620       630       640       650
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1            TGTGGAACACCGCGATGTTTGTGACTGAGACCCCTAATTCGCTGACGCCTGGCTCCTCCTCCATTGTCTATGGCAGTACAACAGTTTCCTCCTAATCGGTTAAGTCTATTCTATCCCACGTTTGTGTTCT
Pre-NTP2            ----------------------------------------------------------------------------------------------------------------------------
Pre-NTP3            ..........................................A.....................................................................
```

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                660       670       680       690       700       710       720       730       740       750       760       770       780
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1   TTAGTTTGAAATGACGCCGGTTTCGGTTCTGCAAGTTTGTAGTGTCGTCGTTGCAGCACAAGGCATGTGATCTACTCGGCTGTAGTGCCCCGCCATGCGAACCGCTCGTTCACACTTGGTAGCATACTAT
Pre-NTP2   ------------------------------------------------------------------------------------------------------------------------------
Pre-NTP3   ..........................................................G.....................................................................

                790       800       810       820       830       840       850       860       870       880       890       900       910
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1   CAACGGCAGAACACGAGAGGAAAACCAATTATTTCCCCATTCACCATCATTCGCCATCACAGCGCTCCGTGTCGTAGCGTGGAGACGATGGCGGCACAAGGCATCCTCAGAACTTTTGTCGTTCTGTTGC
Pre-NTP2   -------C...A...C.GT.T.G.GGC..CC.CCT.G.GGGGC....TC.A-------CAGAA..AAAAG.G..A.T.AAC.GTG.ACGCCAC..CGGC.....................A.........
Pre-NTP3   ..................................................................................................................................

                920       930       940       950       960       970       980       990       1000      1010      1020      1030      1040
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1   ATGGGCGTCAACCTCTGCCTATTCTAGCGCGGGACCACAGTTGCGGGTTCTACGGGGACCCGGCGGCCGAACGTTCACCACGGCAATTGAGACGCACGCATCGTTTATGCGACACCAGCACTCTCTGCCG
Pre-NTP2   ......A...G...T.................T.G.....................................................A.......C.........T...........GGA
Pre-NTP3   ..............................T.....................................................................................................

                1050      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1   ATTGAGACGCATTGCTGAACTCCC----------------------AATGAGACGCGTCCAGAACGAACCCGATCCCGTTTCCGGGGCCCAGCGACGACATTCGGGGCACCGTTAACGTCGCCCAGCG
Pre-NTP2   .G-------------------..TATTGAGACGCATTGCTGAACCCTC.T.................A.------A.....................................................
Pre-NTP3   ............A.........AATGAGACGCATTGCTGAACCCTC.T.....................................................G.....................C

                1180      1190      1200      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pre-NTP1   CCTCGTGTGAGACTGCCACTTGTTCCTGCTGTTCCTGCACGCCCTGTGGTCTATTCCTCATTGTCTCGGTTCCCGGTAGAACTAGGTTCGAGTCCCAGTTTTTTGAGAGTTACTAGAGAGTTGCCTGAGT
Pre-NTP2   ..................G.....................................................................................................
Pre-NTP3   ..................G.....................................................................................................

                1310      1320      1330      1340      1350
           ....|....|....|....|....|....|....|....|....|....|
Pre-NTP1   TACCTTGTCGTTCTGCATCTTCCTGTCTGTTGCCCGTATTCAAGTAGCCACGAA
Pre-NTP2   ..........................................A.........A
Pre-NTP3   ..........................................A.........A
```

OBJETIVO 3/OBJECTIVE 3

Evaluación de NcROP40 y NcNTPasa como candidatos vacunales en formulaciones monovalentes y polivalentes combinadas con las proteínas NcROP2Fam-1 y NcGRA7 en un modelo murino de neosporosis

Evaluation of the usefulness of NcROP40 and NcNTPase-based vaccines in single and polyvalent formulations combined with the NcROP2Fam-1 and NcGRA7 proteins in a mouse model of neosporosis

A vaccine formulation combining rhoptry proteins NcROP40 and NcROP2Fam-1 improves pup survival in a pregnant mouse model of neosporosis

Resumen

Hasta la fecha no se ha desarrollado ningún tratamiento ni vacuna eficaz frente a la neosporosis bovina, por lo que su control se basa en el diagnóstico y en medidas de manejo concretas. No obstante, la vacunación se ha establecido como una medida rentable para el control de la enfermedad. En este sentido, las vacunas de subunidades se presentan como las alternativas más seguras y económicamente viables, si bien su tasa de protección ha sido reducida de acuerdo a los resultados descritos en estudios preliminares. En este sentido, en los últimos años se han evaluado diferentes vacunas basadas en diversos antígenos inmunodominantes o proteínas implicadas en la adhesión e invasión de *N. caninum* en modelos experimentales murinos. Entre ellos destacan las vacunas basadas en las proteínas NcROP2Fam-1 y NcGRA7, cuyo empleo ha aportado los resultados más prometedores. De acuerdo con nuestros estudios, las proteínas NcROP40 y NcNTPasa podrían ser candidatos vacunales relevantes por su papel en el ciclo lítico del taquizoíto, así como por su asociación con la virulencia de *N. caninum*.

En el presente objetivo se evaluó la utilidad vacunal de las proteínas recombinantes NcROP40, NcROP2Fam-1, NcGRA7 y NcNTPasa formuladas con el adyuvante Quil-A como preparados monovalentes o polivalentes (rNcROP40+rNcROP2Fam-1 y rNcGRA7+rNcNTPasa). Dichas formulaciones se probaron en un modelo murino gestante y no gestante de neosporosis, donde se evaluó la seguridad, la respuesta inmunitaria de base humoral y celular, así como su eficacia mediante la evaluación de parámetros reproductivos, clínicos y de transmisión, tanto en las ratonas adultas como en su descendencia.

De acuerdo al sistema de puntuación desarrollado en el presente trabajo, todas las formulaciones fueron seguras y no generaron efectos secundarios graves tras su inoculación. En cuanto a su eficacia frente a la

neosporosis, la mayoría de los preparados vacunales incrementaron el tiempo medio de supervivencia de las crías, aunque sólo se registraron supervivientes en los grupos vacunados con la combinación de las proteínas rNcROP40 y rNcROP2Fam-1 (16,2% de supervivencia) y con la proteína rNcROP2Fam-1 (6,3% de supervivencia). Además, no se detectó transmisión vertical en ninguna de las crías supervivientes del grupo vacunado con rNcROP40+rNcROP2Fam-1, confirmando cierto grado de control en la transmisión del parásito a la progenie. Todas las vacunas indujeron una respuesta inmunitaria de tipo celular y humoral específica frente a sus respectivas proteínas nativas. Concretamente, la vacunación con rNcROP40 y rNcROP40+rNcROP2Fam-1 se asoció con niveles más elevados de IFN-γ tras la inmunización, así como unas cargas parasitarias y unos niveles de IgG más bajos tras el desafío.

El presente objetivo pone de manifiesto que la vacunación con rNcROP40+rNcROP2Fam-1 confiere una protección parcial frente a la infección por *N. caninum* en el modelo murino, y sugiere un efecto sinérgico de la combinación de ambas proteínas en una misma formulación.

A vaccine formulation combining rhoptry proteins NcROP40 and NcROP2Fam-1 improves pup survival in a pregnant mouse model of neosporosis

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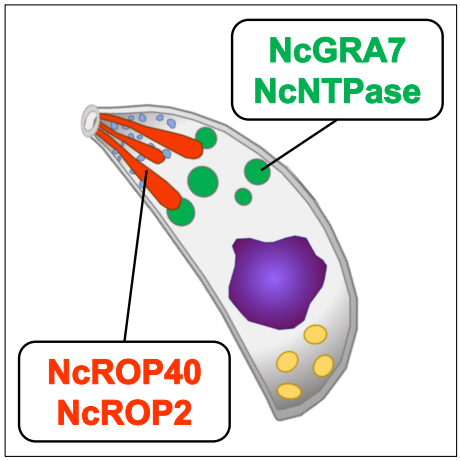

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ABSTRACT

Currently there are no effective vaccines for the control of bovine neosporosis. During the last years several subunit vaccines based on immunodominant antigens and other proteins involved in adhesion, invasion and intracellular proliferation of *Neospora caninum* have been evaluated as targets for vaccine development in experimental mouse infection models. Among them, the rhoptry antigen NcROP2Fam-1 and the immunodominant NcGRA7 protein have been assessed with varying results. Recent studies have shown that another rhoptry component, NcROP40, and NcNTPase, a putative dense granule antigen, exhibit higher expression levels in tachyzoites of virulent *N. caninum* isolates, suggesting that these could be potential vaccine candidates to limit the effects of infection. In the present work, the safety and efficacy of these recombinant antigens formulated in Quil-A adjuvant as monovalent vaccines or pair-wise combinations (rNcROP40+rNcROP2Fam-1 and rNcGRA7+rNcNTPase) were evaluated in a pregnant mouse model of neosporosis. All the vaccine formulations elicited a specific immune response against their respective native proteins after immunization. Mice vaccinated with rNcROP40 and rNcROP2Fam-1 alone or in combination produced the highest levels of IFN-γ and exhibited low parasite burdens and low IgG antibody levels after the challenge. In addition, most of the vaccine formulations were able to increase the median survival time in the offspring. However, pup survival only ensued in the groups vaccinated with rNcROP40+rNcROP2Fam-1 (16.2%) and rNcROP2Fam-1 (6.3%). Interestingly, vertical transmission was not observed in those survivor pups immunized with rNcROP40+rNcROP2Fam-1, as shown by PCR analyses. These results show a partial protection against *N. caninum* infection after vaccination with rNcROP40+rNcROP2Fam-1, suggesting a synergistic effect of the two recombinant rhoptry antigens.

1. Introduction

Bovine neosporosis is one of the major parasitic diseases worldwide leading to reproductive failure in cattle (Dubey & Schares, 2011) where it causes significant economic losses (Reichel *et al.*, 2013). To date, there are no available drugs to prevent *Neospora caninum*-associated vertical transmission and abortion. Vaccination has been described as the most cost-efficient strategy to control neosporosis in cattle (Reichel & Ellis, 2009). Compared to live vaccines, the development of recombinant subunit vaccines represents a desirable option, since they are more stable, and easier and less expensive to produce. Particularly, proteins located in micronemes, rhoptries and dense granules, organelles whose contents are involved in tachyzoite host cell adhesion and invasion, have emerged as promising vaccine targets to block parasite dissemination and foetal transmission during the acute phase of infection (Hemphill *et al.*, 2013). Vaccine candidates have been traditionally selected based on their immunogenic capacities (Ellis *et al.*, 2008), their role in tachyzoite to bradyzoite stage conversion (Uchida *et al.*, 2013; Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a) and their implication in adhesion or invasion processes (Monney *et al.*, 2011; Debache *et al.*, 2009), with variable results in experimental mouse models. Recently, proteome expression changes in different isolates revealed that the rhoptry protein NcROP40 and the dense granule protein NcNTPase are more abundantly expressed in virulent isolates compared to isolates of low virulence (Regidor-Cerrillo *et al.*, 2012). By analogy to other apicomplexan parasites, these proteins may perform relevant functions related to host cell invasion and intracellular

proliferation. Thus, we hypothesized that both NcROP40 and NcNTPase represent relevant targets for vaccination.

Previous works have shown that the efficacy of a monovalent recombinant vaccine based on a single antigen was considerably lower compared to a polyvalent vaccine consisting of a combination of antigens (Dziadek *et al.*, 2012; Dziadek *et al.*, 2011; Debache *et al.*, 2009; Dziadek *et al.*, 2009). Thus, recombinant NcROP40 and NcNTPase proteins were combined with previously assessed recombinant antigens. On the one hand, NcROP2Fam-1 -the first rhoptry protein described in *N.caninum* (Alaeddine *et al.*, 2013)- was co-administred with NcROP40. Both have been described as rhoptry pseudokinases (Talevich & Kannan, 2013), and are members of the ROP2 family. Some members of this family were shown to play an important role as virulence factors in *Toxoplasma gondii* (Boothroyd & Dubremetz, 2008; El Hajj *et al.*, 2006). Furthermore, vaccination of mice with recombinant NcROP2Fam-1 was shown to protect mice against neosporosis (Monney *et al.*, 2011; Debache *et al.*, 2010; Debache *et al.*, 2009; Debache *et al.*, 2008). On the other hand, NcNTPase was shown to form a complex with the immunodominant dense granule antigen NcGRA7, whose efficacy as a recombinant vaccine was also assessed in mice (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a). In *T. gondii* tachyzoites, the expression of the two dense granule antigens TgGRA7 and TgNTPase is essential for active replication (Cesbron-Delauw *et al.*, 2008; Coppens *et al.*, 1999). Accordingly, the objective of the present work was to evaluate the efficacy of monovalent recombinant NcROP40, NcROP2Fam-1, NcGRA7 and NcNTPase vaccines. In addition, combinations of rNcROP40+rNcROP2Fam-1 and rNcGRA7+rNcNTPase were assessed. All recombinant vaccines were formulated in Quil-A saponin, and vaccine trials were performed in a well-established pregnant mouse model of neosporosis. Immunogenicity, safety, and efficacy of these vaccines were evaluated in order to determine their potential immunoprophylactic usefulness against neosporosis.

2. Materials and methods

2.1. Recombinant protein cloning and sequencing

Nc*ROP40* (previously named as NcROP8 with accession number NCLIV_012920 in ToxoDB v. 10), Nc*ROP2Fam-1* (NCLIV_001970 in ToxoDB v. 10), Nc*NTPase* (NCLIV_068400 in ToxoDB v. 10) and Nc*GRA7* (NCLIV_021640 in ToxoDB v. 10) were cloned in the pET45b(+) expression system (Novagen). Primer sequences, restriction enzymes employed and annealing temperatures for PCR are summarised in Supplementary table 1. All primers were purchased from Sigma-Aldrich, and Expand High Fidelity Plus PCR System (Roche) was used for all PCR. Restriction enzymes, alkaline phosphatase and T4 ligase were purchased from New England Biolabs. Amplicons and digested products were purified with the GENECLEAN Turbo kit (MP Biomedicals) from low melting agarose gels (Lonza).

rNcROP40 was obtained in *Escherichia coli* after deletion of 129 pb in the N-terminal sequence from the original construction (Regidor-Cerrillo *et al.*, 2012) in order to increase the efficiency of immobilized metal ion affinity chromatography (IMAC) purification. For this purpose, the pET45-ROP8 plasmid was digested with *Kpn*I, treated with Klenow enzyme, digested with *Sal*I, treated with Mung Bean enzyme and finally re-ligated with a T4 ligase. Klenow and Mung Bean exonucleases were also purchased from New England Biolabs. Nc*ROP2Fam-1* sequence was amplified using as DNA template the pQE30-NcROP2Fam-1 plasmid (Debache *et al.*, 2008). Nc*NTPase* was amplified from Nc-Liv cDNA, and

Nc*GRA7* was cloned as previously described (Jiménez-Ruiz *et al.*, 2012; Álvarez-García *et al.*, 2007).

E. coli NovaBlue Single Competent Cells (Novagen) were transformed with resulting plasmids, which were isolated using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced in the Genomics Unit of the Scientific Park of Madrid with an ABI Prism 377 DNA sequencer (Applied Biosystems) using T7 forward and reverse primers. All sequences were aligned with 100% consensus. Finally, *E. coli* BL21(DE3)pLysS competent cells (Agilent Technologies) were transformed with plasmids from each protein.

2.2. Expression and purification of recombinant proteins

E. coli BL21(DE3)pLysS competent cells (Agilent Technologies) were transformed with the resulting expression vectors and foreign expression of rNcROP40, rNcROP2Fam-1 and rNcNTPase as a $(His)_6$-tagged fusion proteins was carried out following standard procedures (Álvarez-García *et al.*, 2007). Transformed bacteria were cultured in selective medium (Luria Bertoni supplemented with 10 g/l glucose, 100 mg/ml ampicillin and 34 mg/ml chloramphenicol) until the OD_{600} reached 0.6. Recombinant protein synthesis was initiated then by adding isopropyl-β-D-thiogalactopyranoside (IPTG, Calbiochem) to the culture at a final concentration of 1 mM. After incubation (4 h, 37° C), IPTG-induced bacteria were harvested (3500 × *g*, 15 min), resuspended in an isolation buffer containing 2 M urea (Sigma-Aldrich), 20 mM Tris-HCl (Panreac), 0.5 M NaCl (Panreac), 2% Triton X-100 (Merck), lysozyme (10 kU/g crude extract, Novagen), protease inhibition cocktail (50 µl/g crude extract, Sigma-Aldrich) and Benzonase® nuclease (25 U/ml, Sigma-Aldrich), and then lysed by ultrasound treatment in an ice-bath (Branson Sonifier 450, Branson Ultrasonic Co.). Proteins were extracted from inclusion bodies by centrifugation at 16000 × *g* for 10 minutes and denatured with a binding buffer containing 6 M guanidine hydrochloride (Sigma-Aldrich), 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole (Merck) and 1 mM 2-mercaptoethanol (BioRad Laboratories). Then, recombinant proteins were solubilized on-column with a solubilization buffer containing 6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 20% glycerol (Sigma-Aldrich) and 1 mM 2-mercaptoethanol using HisTrapHP columns coupled to the ÄKTAprime Plus system (GE Healthcare). Afterwards, proteins were on-column refolded based on a descent gradient of the chaotrophic agent with a refolding buffer containing 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 20% glycerol, 5% glucose (Panreac) and 1 mM 2-mercaptoethanol. Finally, proteins were obtained by elution with a buffer containing 20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole, 20% glycerol, 5% glucose and 1 mM 2-mercaptoethanol, and stored at -80° C until use. The recombinant NcGRA7 (rNcGRA7) was produced as a soluble protein and purified by IMAC as previously described (Álvarez-García *et al.*, 2007). All proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to check their purity and integrity. Protein concentration was measured using a GS-800™ Calibrated Densitometer coupled to the Quantity One software v.4.69 (Bio-Rad Laboratories) using a standard BSA scale (Roche). All the proteins were stored at -80ºC until use.

2.3. Vaccine preparation

Quil-A purified saponin (Accurate Chemical & Scientific Corp.) was resuspended in PBS and 0.2 µm-filtered. A total of 25 µg of each recombinant proteins were directly mixed with 10 (first inoculation) or 5 (second and third inoculation) µg of Quil-A on sterile PBS in a final volume of 200 µl (Table 1). All the

immunisations were conducted with the same batch of Quil-A. Formulations were prepared immediately and their limpidity was checked prior to inoculations.

2.4. Parasite culture

N. caninum tachyzoites of the Nc-Liverpool isolate (Barber *et al.*, 1993) were grown *in vitro* using standard procedures by continuous passage in MARC-145 cell culture maintained in Dulbecco's Modified Eagle Medium (DMEM, Lonza). For the challenge, tachyzoites were harvested by centrifugation, passed through a 21-gauge needle, resuspended in culture medium at the required dose (2×10^6 in a final volume of 200 µl per mouse) and used immediately to infect the mice. The number of viable tachyzoites was estimated by Trypan blue exclusion (Collantes-Fernández *et al.*, 2004).

Table 1: Immunized groups

Group	*n*	Protein	Dose[c]	Adjuvant	Challenge[d]
1	20[a]+5[b]	rNcROP40	25 µg	Quil-A	2 x 10^6
2	20[a]+5[b]	rNcROP2Fam-1	25 µg	Quil-A	2 x 10^6
3	20[a]+5[b]	rNcROP40+rNcROP2Fam-1	25 µg+25 µg	Quil-A	2 x 10^6
4	20[a]+5[b]	rNcGRA7	25 µg	Quil-A	2 x 10^6
5	20[a]+5[b]	rNcNTPase	25 µg	Quil-A	2 x 10^6
6	20[a]+5[b]	rNcGRA7+rNcNTPase	25 µg+25 µg	Quil-A	2 x 10^6
7	10[a]+5[b]	-	-	Quil-A	2 x 10^6
8	10[a]+5[b]	-	-	-	PBS
9	10[a]	-	-	-	2 x 10^6

[a] Mice mated and challenged with Nc-Liv tachyzoites
[b] Mice non-mated and non-challenged to study the elicited immune response after vaccination
[c] Amount of protein(s) inoculated per mouse for each inoculation
[d] Dose of Nc-Liv tachyzoites inoculated per mouse

2.5. Vaccine trials: experimental design, sampling and data collection

All mice handling procedures complied with current EU legislation and were approved by the Ethics Commitee of the Complutense University. Eight week-old female BALB/c AnNCrl mice (Charles River Laboratories) were randomly divided into nine groups and inoculated subcutaneously three times at 3-week intervals (see Table 1). Safety of vaccine formulations was determined as clinical side effects associated with immunization during the next week after each inoculation. These effects were assessed daily according to two different scores established by systemic clinical signs and the appearance of nodules at the point of inoculation (Table 2).

In order to analyse humoral and cellular immune responses elicited by the tested formulations, five females from groups 1 to 8 were euthanized in a CO_2 chamber five days after the last booster, before mating and challenge. Serum samples were collected by cardiac puncture for serological analysis and spleens were aseptically extracted and immediately processed for splenocyte culture to quantify IFN-γ and IL-4 production.

In the remaining females, pregnancy was achieved following procedures previously described (López-

Pérez *et al.*, 2006). Briefly, 2 weeks after the third inoculation, mice were mated for 96 h following oestrus synchronization by the Whitten effect (Whitten, 1957). Then, mice from groups 1 to 7 and 9 were challenged subcutaneously with 2×10^6 tachyzoites of Nc-Liverpool isolate at mid gestation (days 8 to 11 of gestation) while mice from group 8 were left unchallenged and received a PBS inoculation (Table 1). Dams and their progeny were evaluated from birth to day 30 postpartum (p.p.). Data on the fertility rate (percentage of female mice housed with males that became pregnant), litter size (number of born pups), early pup mortality (number of full-term dead pups from birth until day 2 p.p.), post-natal mortality (number of dead pups from day 3 to day 30 p.p.), and clinical signs of dams and non-pregnant mice were recorded during this time. Clinical signs were scored according to general appearance of mice and the presence of clinical signs compatible with neosporosis such a ruffled coat, rounded back, low body weight and nervous signs (Table 2). Dams, non-pregnant mice and surviving pups were euthanized in a CO_2 chamber on day 30 p.p. (approximately day 50 post-challenge, p.c.). Sera from dams and non-pregnant mice were recovered to test humoral immune responses. Brains from dams, non-pregnant mice and survival pups were also sampled for parasite detection and quantification by PCR. Collected tissue samples were frozen at -20º C until further analysis.

Table 2: Scores of vaccine safety and efficacy

Score	Vaccine safety		Vaccine efficacy
	Systemic signs	*Nodules*	*Clinical signs*
0	No alterations	Absent	No signs
1	Ruffled coat	Small size (< 2.5 mm)	Ruffled coat
2	Ruffled coat + activity decrease < 24 h	Medium size (< 5 mm)	Ruffled coat + rounded back
3	Ruffled coat + activity decrease < 48 h	Large size (< 10 mm)	Ruffled coat + rounded back + low body weight (> 20% weight loss)
4	Ruffled coat + activity decrease > 48 h	Extra large size (> 10 mm)	Ruffled coat + rounded back + low body weight + nervous signs *

* All animals showing neurological signs were euthanized for humanitarian reasons

2.6. Analysis of humoral immune response

N. caninum-specific serum isotypes IgG1 and IgG2a were determined by ELISA in dams and non-pregnant mice using a soluble *N. caninum* tachyzoite antigen and anti-mouse IgG1 or IgG2a peroxidase-conjugated as secondary antibody as previously described (Marugán-Hernández *et al.*, 2011b).

Recombinant proteins and whole tachyzoite extracts were run in 15% SDS-PAGE gels and then transferred onto nitrocellulose membranes for immunoblotting analysis of mice sera, following standard procedures previously described (Jiménez-Ruiz *et al.*, 2012; Álvarez-García *et al.*, 2002).

2.7. Analysis of cellular immune response: IFN-γ and IL-4

IFN-γ and IL-4 levels from groups 1 to 8 were measured in stimulated splenocyte culture supernatants as previously described (Jiménez-Ruiz *et al.*, 2012) through a commercial ELISA kit (eBioscience) following the supplied protocol. Standard curves were adjusted following a logistic regression (5PL model)

with the Reader Fit tool (http://www.readerfit.com, Hitachi).

2.8. PCR detection and quantification of parasite burden in brains

The Maxwell® 16 system was employed to automatically extract DNA from brain samples from adult females and survivor pups using the mouse tail DNA purification kit (Promega) according to the manufacturers' protocol. DNA concentration was measured by UV spectrophotometry using a NanoPhotometer System (Implen). Then, PCR detection of parasite DNA was carried out by nested-PCR of the ITS1 region as previously described (Buxton *et al.*, 1998). Pups that succumbed to infection from 3 to 30 days p.p. were not analysed and assumed as PCR-positive, according to previous findings (Dellarupe *et al.*, 2014a).

Parasite burden was quantified in brains from dams and non-pregnant mice by real-time PCR using primers and conditions as previously described (Rojo-Montejo *et al.*, 2011a). Parasite load was expressed as parasite number/µg host DNA.

2.9. Statistical analysis

Data analysis was performed following previous recommendations (Morrison, 2002). Differences in fertility rates, mortality, morbidity and parasite presence in brains were analyzed by Chi-square (χ^2) and Fisher *F*-tests. Post-natal mortality was analyzed by the Kaplan-Meier survival method (Bland & Altman, 1998). The Log-rank statistical test was applied to compare the survival curves between the different groups (Bland & Altman, 2004). For pair-wise comparisons, the 0.05/*k* adjustment was applied to the *P* value, where *k* corresponded to the number of groups. One-way ANOVA followed by Tukey's multiple range test were employed to compare anti-*N. caninum* antibody levels, cytokine levels and litter size. Unpaired two tailed *t*-test was used for comparisons between IgG1 and IgG2a levels within each group. Parasite burdens were analysed using the Kruskal–Wallis test followed by Dunn's test for comparisons between groups. Parasite burden comparison between dams and non-pregnant mice was analysed using the *U* Mann-Whitney test. All statistical analyses were carried out using GraphPad Prism 5 v.5.01 software.

3. Results

3.1. Expression of recombinant proteins

rNcROP40, rNcROP2Fam-1, rNcGRA7 and rNcNTPase migrated at approximately 39, 38, 30 and 64 kDa, respectively (Supplementary Figure 1), according to the expected apparent molecular mass.

3.2. Induction phase (pre-challenge)

3.2.1. Vaccine safety

Clinical side effects related to vaccination were recorded during the experiment (Supplementary Figure 2A). Mice from rNcGRA7 and rNcROP40+rNcROP2Fam-1-vaccinated groups exhibited ruffled coat for 24 h (score = 1), which could be associated to fever. Mice from the rNcGRA7+rNcNTPase-group also showed a decrease in their normal activity for 24 h (score = 2). The other groups remained clinically unaffected by the vaccination procedures. Local skin reactions (nodules) at the inoculation site were detected in all immunized groups. rNcGRA7+rNcNTPase and rNcROP40+rNcROP2Fam-1-vaccinated mice were most seriously affected (score = 2-3 and 1-2, respectively), whilst the individual score in the other groups did not surpass the value of 1 (Supplementary Figure 2B). All nodules had disappeared at the end of the experiment.

3.2.2. Humoral immune response

Vaccination of mice with all recombinant antigen preparations elicited specific humoral responses against soluble tachyzoite extracts, as shown by ELISA (Figure 1A). Specifically, rNcGRA7 and rNcGRA7+rNcNTPase-vaccinated groups exhibited the most pronounced IgG1 responses among the vaccinated groups ($P < 0.05$; one-way ANOVA). With regard to IgG2a synthesis, the rNcROP2Fam-1-vaccinated group displayed the lowest responses in comparison to the groups vaccinated with rNcROP40, rNcGRA7, rNcNTPase and rNcGRA7+rNcNTPase ($P < 0.05$; one-way ANOVA) (Figure 1A).

Comparison of IgG1 and IgG2a levels within groups revealed a IgG1-biased immune response in groups vaccinated with rNcROP2Fam-1, rNcGRA7, rNcROP40+rNcROP2Fam-1 and rNcGRA7+rNcNTPase ($P < 0.05$, *t*-test). However, comparison of the IgG1/IgG2a ratios between the groups did not reveal significant differences ($P > 0.05$; one-way ANOVA) (Figure 1A).

Mouse sera were analysed by Western-blotting of recombinant antigens and of whole tachyzoite extracts. The sera reacted specifically with the respective recombinant protein band(s) used for vaccination (Supplementary Figure 1). In addition, all sera contained antibodies that labelled their corresponding original proteins in tachyzoite extracts (Figure 1B), as previously described in different studies. Specifically, sera from rNcROP40-vaccinated mice recognized bands of 53, 29 and 25 kDa (Regidor-Cerrillo *et al.*, 2012), while sera from mice vaccinated with rNcROP2Fam-1 labelled bands of 64, 55 and 49 kDa (Alaeddine *et al.*, 2013). Sera from mice vaccinated with rNcGRA7-group stained bands of 33 and 18 kDa (Álvarez-García *et al.*, 2006), and mice vaccinated with rNcNTPase-elicited a humoral immune response against a 67 kDa band (Asai *et al.*, 1998). Furthermore, mice inoculated with rNcROP40+rNcROP2Fam-1 or rNcGRA7+rNcNTPase recognized a mixture of their corresponding protein bands.

3.2.3. Cellular immune responses

Cellular immune responses were measured by splenocyte stimulation with either *N. caninum* tachyzoite extracts or recombinant antigen preparations and quantification of IFN-γ and IL-4 levels in culture supernatants by ELISA. After stimulation with *N. caninum*-extract, only cells from mice vaccinated with rNcROP2Fam-1 secreted significantly increased amounts of IFN-γ in comparison with the remainder groups ($P < 0.05$; one-way ANOVA) (Figure 1C). Furthermore, splenocytes from rNcROP40, rNcROP40+rNcROP2Fam-1 and rNcGRA7-vaccinated mice secreted lower amounts (10 to 30 ng/ml) of IFN-γ upon stimulation with *N. caninum*-extract. In contrast, IFN-γ levels in culture supernatants obtained from splenocytes of mice vaccinated with rNcNTPase and rNcGRA7+rNcNTPase remained below 2 ng/ml, and those from non-vaccinated mice were below 1 ng/ml. However, these differences were not statistically significant ($P > 0.05$; one-way ANOVA). A similar profile was detected after stimulation with recombinant antigens. The highest levels for secretion of IFN-γ were detected in the rNcROP2Fam-1-group ($P < 0.05$; one-way ANOVA) (Figure 1C). In contrast, splenocytes of mice vaccinated with rNcGRA7, rNcNTPase and rNcGRA7+rNcNTPase displayed similar levels of IFN-γ expression to those of unvaccinated-groups ($P > 0.05$; one-way ANOVA).

No significant differences in IL-4 expression were detected after stimulation with both *N. caninum*-extract and recombinant proteins in any of the groups ($P > 0.05$; one-way ANOVA). Specifically, IL-4 values did not surpass 0.2 ng/ml, neither in vaccinated nor in non-vaccinated groups (Figure 1C).

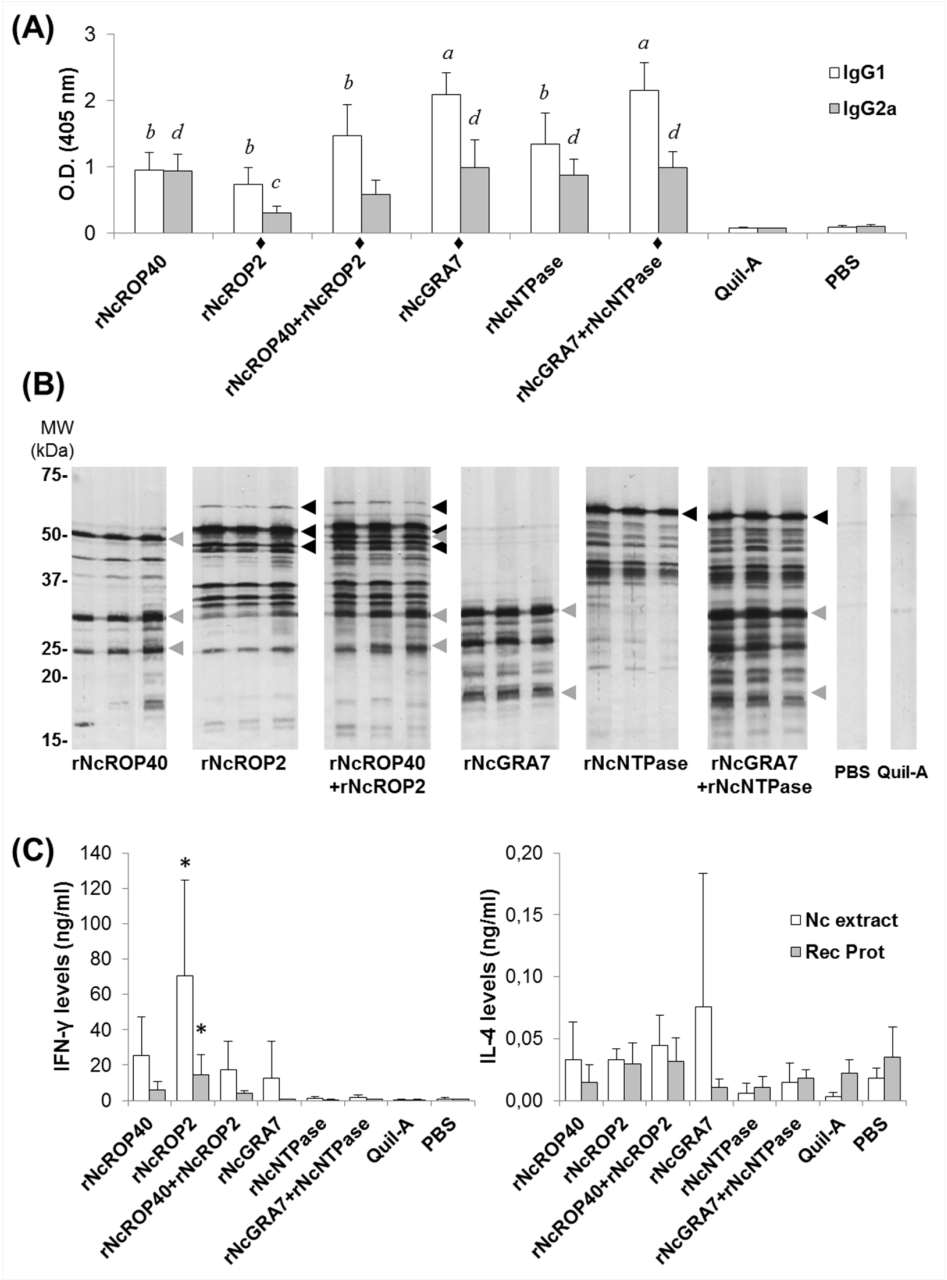

Figure 1: Immune responses in vaccinated mice during the induction phase. (A) Anti-*N. caninum* immunoglobulins (IgG1 and IgG2a isotypes) generated against tachyzoite soluble extract after vaccination. Bars represent the average optical density (O.D.) and error bars represent standard deviations for each group. ***a***, ***b*** and ***c***, ***d*** indicate significant differences ($P < 0.05$, one-way ANOVA). ✦ shows differences between IgG1 and IgG2a levels within each group ($P < 0.05$; *t*-test). (B) *N. caninum* tachyzoite-based Western-Blot from vaccinated mice before challenge. Head arrows indicate the recognition of original proteins by mice sera. rNcROP40-group recognized bands of 53, 29 and 25 kDa; rNcROP2Fam-1-group recognized bands of 64, 55 and 49 kDa; rNcGRA7-group recognized bands of 33 and 18 kDa; rNcNTPase-group recognized a band of 67 kDa and rNcROP40+rNcROP2Fam-1 and rNcGRA7+rNcNTPase-groups recognized a mixture of the mentioned bands for each case. (C) Secretion of IFN-γ (left) and IL-4 (right) by splenocytes of vaccinated mice during the pre-challenge phase after *in vitro* stimulation with *N. caninum* extract and recombinant protein(s) (see legend). * indicates the highest levels of secretion among groups ($P < 0.05$, one-way ANOVA).

3.3. Effector phase (post-challenge)

3.3.1. Vaccine efficacy in dams

Concerning morbidity, skin lesions at the site of parasite inoculation were observed in all challenged groups between days 7 and 14 post infection (p.i.). Dams from vaccinated and challenged groups exhibited clinical signs such as ruffled coat (rNcROP2Fam-1, rNcROP40+rNcROP2Fam-1, rNcGRA7, rNcNTPase; score = 1) and rounded back (rNcROP40 and rNcGRA7+rNcNTPase; score = 2). In contrast, unvaccinated and challenged groups exhibited more pronounced clinical signs, reaching higher scores (score = 3).

Data on fertility rates, mortality and parasite presence are summarised in Table 3. After challenge, no mortality was observed in any of the vaccinated groups, with the exception of one dam immunized with rNcROP40. This mouse maintained good body condition and did not develop apparent neurological signs, although parasite DNA was detected in its brain. In addition, two dams vaccinated with rNcROP40 and rNcROP2Fam-1, respectively, were euthanized due to partum complications. Regarding fertility, rNcGRA7+rNcNTPase showed the lowest rate after pair-wise comparisons, although differences were non-significant after 0.05/*k* adjustment.

Cerebral parasite infection was detected in over 83% of the dams in all groups. rNcROP2Fam-1 and rNcROP40-groups displayed the lowest rates of PCR detection ($P > 0.05/k$; χ^2). In addition, no differences on parasite burden were found among groups ($P > 0,05$; Kruskal-Wallis *H*-test), although mean values were higher in groups vaccinated with rNcGRA7 and rNcGRA7+rNcNTPase and lower in mice vaccinated with rNcROP40+rNcROP2Fam-1 (Supplementary Figure 3).

3.3.2. Humoral immune responses in dams

As expected, humoral immune responses measured by a tachyzoite extract based ELISA remained high in all challenged mice, and IgG values were higher than those obtained during the induction phase. In particular, serum IgG1 levels were higher in mice vaccinated with rNcGRA7 and rNcGRA7+rNcNTPase compared to those in mice immunized with rNcROP40 and rNcROP2Fam-1 ($P < 0.05$; one-way ANOVA). With regard to IgG2a, rNcGRA7+rNcNTPase vaccinated mice elicited higher levels than mice receiving rNcROP2Fam-1 ($P < 0.05$; one-way ANOVA) (Figure 2A). In addition, as described during the pre-challenge phase, a predominantly IgG1-biased response was detected within groups in all vaccinated mice ($P < 0.05$; *t*-test). Comparison of IgG1/IgG2a ratios revealed no differences between groups ($P > 0.05$; one-way ANOVA).

Western-Blot analyses demonstrated a differential recognition pattern of tachyzoite antigens among groups after parasite challenge. Both vaccinated and unvaccinated mice showed a specific recognition of the immunodominant tachyzoite antigens. Nevertheless, only sera from vaccinated mice reacted against their corresponding original proteins in each group, whilst these antigens were not detected by sera of unvaccinated mice (Figure 2C). The apparent molecular weights of the differentially recognized bands in the vaccinated mice corresponded with those described during the induction phase (Figure 1B).

3.3.3. Vaccine efficacy in pups

Data on litter size, early pup mortality, post-natal mortality and survival, median survival time and vertical transmission rate for each group are summarized in Table 4.

No significant differences in litter sizes were observed among the groups ($P > 0.05$, one-way ANOVA coupled

to Tukey's comparison test), suggesting that fertility was not noticeably altered by vaccination. Groups vaccinated with rNcROP40, rNcGRA7 and rNcGRA7+rNcNTPase showed the highest early pup mortality rates, showing significant differences with the unchallenged group ($P < 0.05/k$; Fisher's exact test).

High morbidity rates were observed in all challenged groups. Pups displayed a delay in growth and hair coat development, weight losses and nervous signs (ataxia, hind limb paresis and walking in circles). Post-natal mortality from 3 to 30 days p.p. was 100% in the groups vaccinated with rNcROP40, rNcGRA7, rNcNTPase and rNcGRA7+rNcNTPase, while only 6% of pups died early after birth in the unchallenged group ($P < 0.05/k$; Fisher's exact test). When survival curves were analysed, groups vaccinated with, rNcROP40, rNcROP2Fam-1, rNcGRA7, rNcNTPase, and rNcROP40+rNcROP2Fam-1 exhibited a significantly increased median survival time in the offspring compared to the unvaccinated and challenged group ($P < 0.05/k$; Log-rank test). In contrast, the group vaccinated with rNcGRA7+rNcNTPase showed the same median survival time that the unvaccinated and challenged group, despite two pups survived until days 21 and 26 p.p. (Figure 3A and 3B; Table 4). Furthermore, rNcROP2Fam-1 and rNcROP40+rNcROP2Fam-1-vaccinated groups exhibited a survival rate of 6.3 and 16.2%, respectively (Figure 3A). Moreover, the number of surviving pups from mice immunized with the combined rNcROP40+rNcROP2Fam-1 vaccine was statistically higher than in all other groups ($P < 0.05/k$; Log-rank test).

Most of the challenged groups showed 100% of vertical transmission. Interestingly, rNcROP40+rNcROP2Fam-1-group showed the lowest vertical transmission rate (83.8%). The surviving pups from this group were distributed among three different litters and were PCR-negative. In fact, in terms of vertical transmission most of the challenged groups differed to the group vaccinated with rNcROP40+rNcROP2Fam-1 ($P < 0.05$; χ^2), but these differences were not significant after $0.05/k$ adjustment.

3.3.4. Vaccine efficacy and humoral immune responses in non-pregnant mice

Data on mortality and cerebral infection are summarized in Table 3. Similar to pregnant mice, non-pregnant mice infected with *N. caninum* exhibited skin lesions at the site of parasite inoculation between days 7 and 14 p.i. In general, all vaccinated and challenged groups exhibited clinical signs compatible with neosporosis such as ruffled coat (score = 1), but no mortality was recorded. In contrast, non-vaccinated and challenged mice displayed scores between 2 and 3. *Neospora* DNA was detected in brain tissues in over 73% of the mice, with no differences among challenged groups ($P > 0.05$; Kruskal-Wallis *H*-test). Similar results were observed in terms of cerebral parasite burden ($P > 0.05$; Kruskal-Wallis *H*-test). However, highest cerebral parasite loads were observed in rNcGRA7 and rNcGRA7+rNcNTPase-vaccinated groups. In addition, parasite burdens were lower in non-pregnant mice in comparison with dams, although differences were not significant ($P > 0.05$; Mann-Whitney *U*-test).

Levels of IgG1 and IgG2a remained similar in all groups ($P > 0.05$; one-way ANOVA) (Figure 2B). However, IgG1 levels were higher than IgG2a ones when compared within groups ($P < 0.05$; *t*-test). These differences were not detected after comparison of IgG1/IgG2a ratios ($P > 0.05$; one-way ANOVA).

Table 3: Vaccine efficacy in dams and non-pregnant mice

Group	Fertility[a]	Dams		Non-pregnant	
		Mortality[b]	*Parasite presence*[c]	*Mortality*[b]	*Parasite presence*[c]
rNcROP40	70%	1/14 (7%)	13/14 (93%)	0/6 (0%)	6/6 (100%)
rNcROP2Fam-1	65%	0/13 (0%)	10/12 (83%)	0/7 (0%)	7/7 (100%)
rNcROP40+rNcROP2Fam-1	45%	0/9 (0%)	9/9 (100%)	0/11 (0%)	9/11 (82%)
rNcGRA7	45%	0/9 (0%)	9/9 (100%)	0/11 (0%)	8/11 (73%)
rNcNTPase	40%	0/8 (0%)	8/8 (100%)	0/12 (0%)	9/12 (75%)
rNcGRA7+rNcNTPase	25%	0/5 (0%)	5/5 (100%)	0/15 (0%)	14/15 (93%)
Quil-A	80%	0/8 (0%)	8/8 (100%)	0/2 (0%)	2/2 (100%)
PBS-Unchallenged	80%	0/8 (0%)	0/8 (0%)	0/2 (0%)	0/2 (0%)
PBS-Challenged	70%	0/7 (0%)	7/7 (100%)	0/3 (0%)	3/3 (100%)

[a]Percentage of pregnant mice.
[b]No. of dead dams/no. of dams (percentage).
[c]No. of nested-PCR positive dams at day 30 p.p./no. of dams in the group (percentage)

Table 4: Vaccine efficacy in pups

	Litter size	**Early pup mortality**		**Post-natal mortality**		**Survival rate**	**Median survival time**	**Vertical transmission**
		Per pup	*Per litter*	*Per pup*	*Per litter*			
rNcROP40	6.85±1.99	35/83 (42.2%)	10/12 (83.3%)	48/48 (100%)	10/10 (100%)	0/48 (0%)	12	48/48 (100%)
rNcROP2Fam-1	5.42±2.39	17/65 (26.1%)	8/12 (66.6%)	45/48 (93.7%)	10/10 (100%)	3/48 (6.3%)	12	47/48 (98%)
rNcROP40+ rNcROP2Fam-1	5.1±1.54	5/42 (11.9%)	3/9 (33.3%)	31/37 (83.8%)	9/9 (100%)	6/37 (16.2%)	14	31/37 (83.8%)
rNcGRA7	5.55±2.65	21/50 (42%)	7/9 (77.7%)	29/29 (100%)	7/7 (100%)	0/29 (0%)	12	29/29 (100%)
rNcNTPase	6±1.5	9/52 (17.3%)	4/8 (50%)	43/43 (100%)	7/7 (100%)	0/43 (0%)	13	43/43 (100%)
rNcGRA7+ rNcNTPase	7.6±2.3	18/38 (47.4%)	4/5 (80%)	20/20 (100%)	4/4 (100%)	0/20 (0%)	10	20/20 (100%)
Quil-A	6±1.69	10/48 (20.8%)	4/8 (50%)	38/38 (100%)	8/8 (100%)	0/38 (0%)	10	38/38 (100%)
PBS-Unchallenged	7±2.67	6/56 (10.7%)	2/8 (25%)	3/50 (6%)	2/8 (25%)	47/50 (94%)	Undef.	0/50 (0%)
PBS-Challenged	5.57±1.99	9/39 (23.1%)	2/7 (28.6%)	30/30 (100%)	7/7 (100%)	0/30 (0%)	10	30/30 (100%)

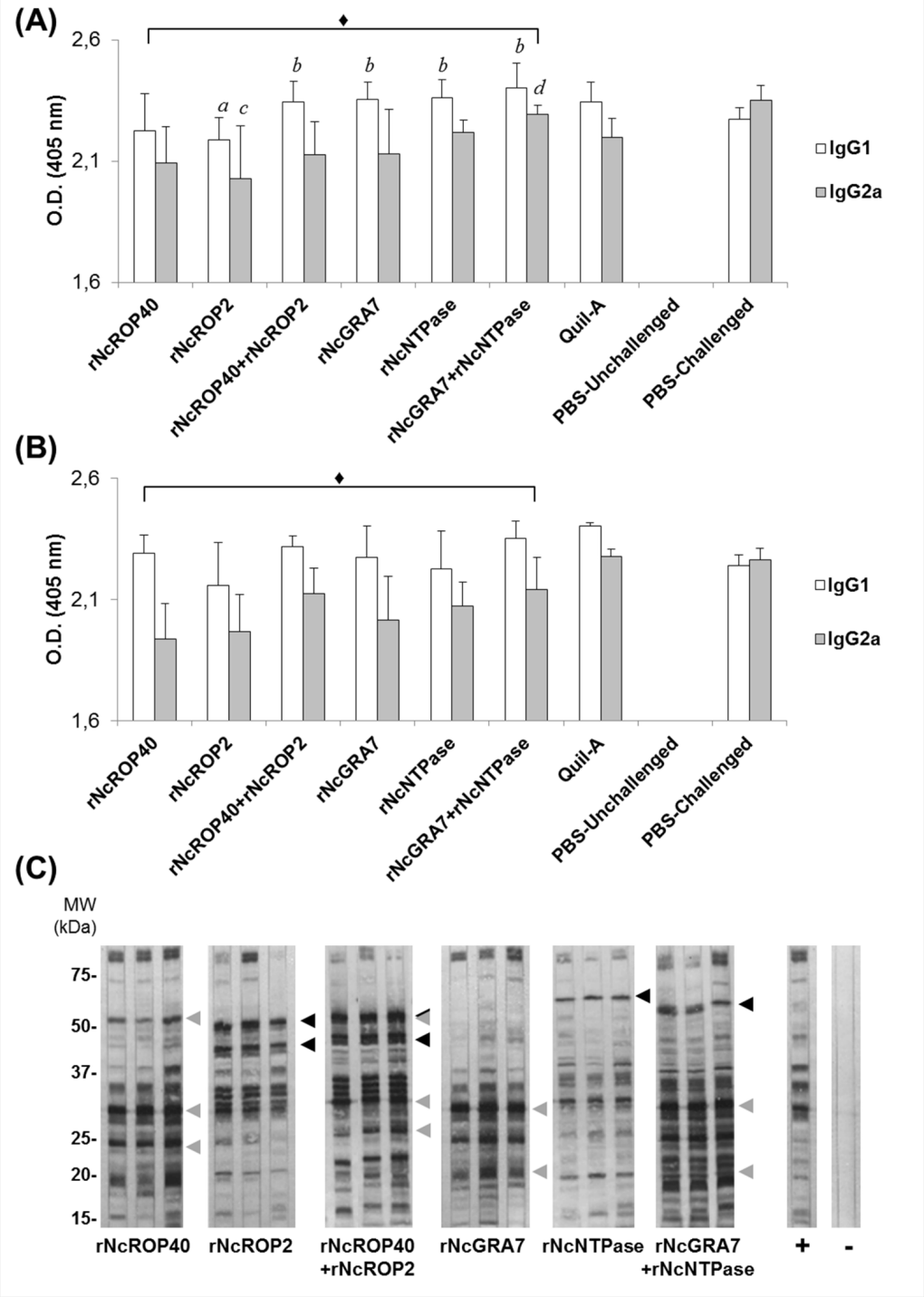

Figure 2: Immune responses in challenged mice during the effector phase. (A) Anti-*N. caninum* immunoglobulins (IgG1 and IgG2a isotypes) generated by vaccinated and unvaccinated dams after challenge. Bars represent the average opitcal density (O.D.), and error bars represent standard deviations for each group. *a*, *b* and *c*, *d* indicate significant differences ($P < 0.05$, one-way ANOVA). ✦ shows statistical differences between IgG1 and IgG2a levels within groups ($P < 0.05$; *t*-test). (B) Anti-*N. caninum* immunoglobulins (IgG1 and IgG2a isotypes) generated by vaccinated and unvaccinated non-pregnant mice after challenge. Bars represent the average optical density (O.D.), and error bars represent standard deviations for each group. ✦ shows statistical differences between IgG1 and IgG2a levels within groups ($P < 0.05$; *t*-test). (C) *N. caninum* tachyzoite-based Western-Blot from vaccinated dams after challenge. Unvaccinated groups are indicated with – (non-challenged) and + (challenged). Head arrows designate protein bands differentially recognized by vaccinated and challenged mice. Bands matched with those observed during the induction phase before challenge, indicating the maintenance of humoral response against vaccine targets after parasite inoculation.

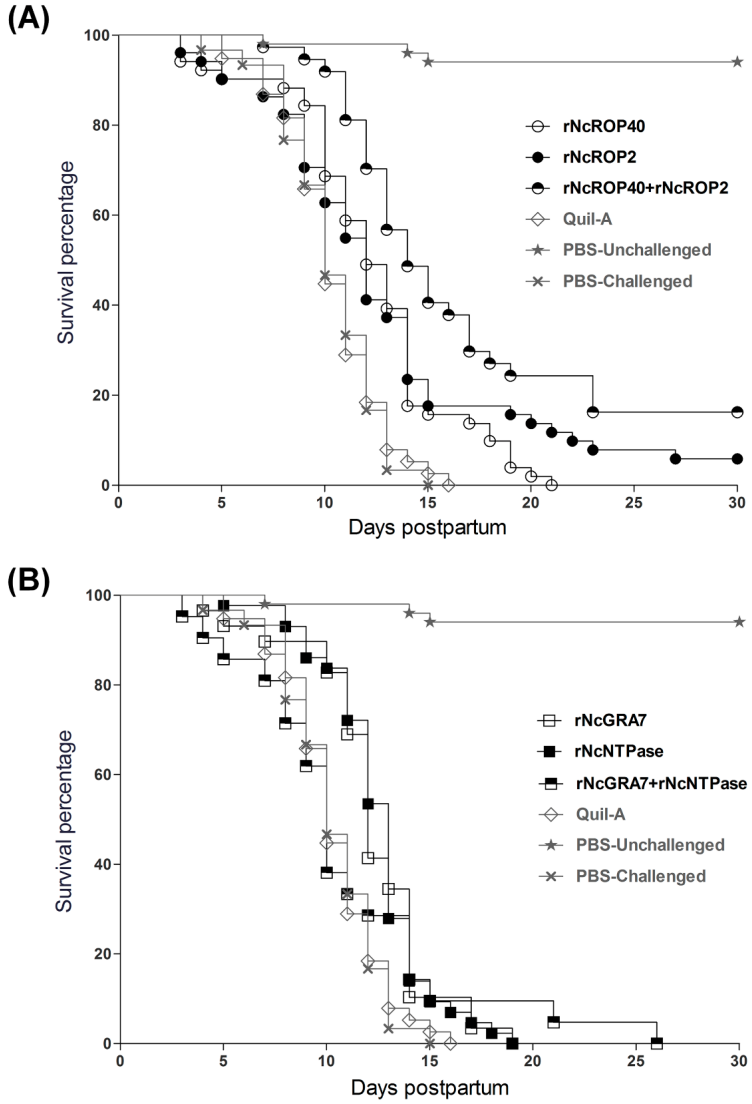

Figure 3: Survival curves from offspring. Kaplan-Meier curves for pups from groups vaccinated with rhoptry proteins (rNcROP40, rNcROP2Fam-1 and rNcROP40+rNcROP2Fam-1; panel A) and dense granules proteins (rNcGRA7, rNcNTPase and rNcGRA7+rNcNTPase; panel B). In both cases unvaccinated groups are shown in grey. Curves represent percent survival as the proportion of all individuals surviving over a period of 30 days after birth and downward steps correspond with observed deaths. Note that a delay in time to death was observed in all vaccinated groups compared to the PBS-challenged and Quil-A group.

4. Discussion

Experimental mouse models have been widely employed as a *proof of concept* to develop vaccines against bovine neosporosis, since they allow a rapid screening of potential vaccine targets before switching to bovine models (Hemphill *et al.*, 2013). In the present work, NcROP40 and NcNTPase, which are more abundantly expressed in high virulence isolates (Regidor-Cerrillo *et al.*, 2012), were expressed as recombinant proteins and assessed as vaccine candidates, either as monovalent vaccines or in combination with NcROP2Fam-1 and NcGRA7 (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a; Debache *et al.*, 2009). Combining these vaccine candidates into a polyvalent formulation was done in anticipation to increase the efficacy of final formulations, since these antigens were selected based on their predicted functional role during the tachyzoite lytic cycle (Cesbron-Delauw *et al.*, 2008; El Hajj *et al.*, 2006; Ferguson, 2004; Coppens *et al.*, 1999). We hypothesized that vaccines against components

of rhoptries or dense granules could elicit an effective immune response to block parasite essential processes and control parasite dissemination.

Since pregnancy success depends on a Th2-biased immune response (Kano *et al.*, 2007; Chaouat *et al.*, 1990), Quil-A was chosen as an adjuvant due to its ability to elicit an adequate Th1/Th2 balance, which would prevent the appearance of immunological disorders during pregnancy. Moreover, it has been approved for veterinary use (Sun *et al.*, 2009). In addition, Quil-A has been widely employed in vaccine formulations against other apicomplexan parasites (Bitencourt *et al.*, 2013; Igarashi *et al.*, 2010; García *et al.*, 2007). In the present work, vaccine safety was evaluated in detail to record possible local adverse reactions at the point of inoculation that have been previously reported for Quil-A adjuvant (Sun *et al.*, 2009). In addition, the general status of vaccinated animals was monitored daily after each inoculation. Both parameters were objectively classified using a scoring system, demonstrating that mice vaccinated with antigen combinations obtained higher scores than those vaccinated with single formulations, indicative of a greater stimulus at the inoculation site in the former. In particular, mice vaccinated with rNcGRA7+rNcNTPase displayed the highest scores, reflecting a combination of two events: the high immunogenicity of NcGRA7 (Aguado-Martínez *et al.*, 2008) and the inoculation of two antigens in each booster. Despite of that, all formulations were proven to be safe since they did not generate severe adverse effects.

Immunogenicity of antigens after vaccination was demonstrated by Western blotting and ELISA, showing that mice developed antibodies against each recombinant protein, and these antibodies also recognized their respective proteins in tachyzoite extracts. Thus, despite the fact that on bacterially expressed antigens conformational epitopes may not be exposed, mice still developed a specific immune response against the vaccine targets in their native form. In addition, we assume that this response remained active after the infection, since sera from vaccinated and challenged animals recognized the same specific bands by Western blotting. Hence, the lack of a protective effect cannot be attributed to a lack of immune stimulation against the original protein as reported in previous studies (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a; Cannas *et al.*, 2003a).

After vaccination with rNcROP2Fam-1, rNcROP40+ rNcROP2Fam-1, rNcGRA7 and rNcGRA7+rNcNTPase, the respective groups elicited higher IgG1 levels, which is in accordance with previous studies (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a; Debache *et al.*, 2008). Despite the marked differences between IgG1 and IgG2a levels in the mice vaccinated with rNcROP40+rNcROP2Fam-1, no alterations were observed during pregnancy. In contrast, those mice immunized with rNcGRA7+rNcNTPase displayed the lowest fertility rate and the highest early pup mortality. The reason for this could be an alteration of the Th1/Th2 balance after vaccination as previously indicated (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a). Nevertheless, this group exhibited the most severe side effects and therefore the high degree of immunological stimulation could have been detrimental for successfully establishing pregnancy already during the mating phase.

Vaccination with rNcROP40+rNcROP2Fam-1, rNcROP40, rNcROP2Fam-1, rNcGRA7 and rNcNTPase slightly prolonged the median survival time of pups from infected dams. Besides, rNcROP2Fam-1 and rNcROP40+rNcROP2Fam-1 inoculation increased the survival rate to 6.3 and 16.2%, respectively. Previous studies carried out under the same experimental conditions but using other vaccine candidates did not achieve survival rates as high as those described in

this study for rNcROP40+rNcROP2Fam-1 (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a). However, rNcGRA7-based vaccines did not protect against pup mortality in combination with Quil-A as shown in the mentioned works, where 8 to 11% of pup survival was described. In contrast, vaccination with rNcROP2Fam-1 in a pregnant mouse model achieved a 50% of pup survival (Debache *et al.*, 2009). Nevertheless, different adjuvants were employed in each study, confirming previous observations with respect to the adjuvant influence on vaccine efficacy (Rojo-Montejo *et al.*, 2011a). In addition, the variable protection observed in this study compared to earlier experiments on rNcROP2Fam-1-based vaccines (Debache *et al.*, 2009) can also be attributed to the use of different isolates (Nc-1 *versus* Nc-Liv) for challenge infection. The rNcROP40+rNcROP2Fam-1-based formulation prevented vertical transmission in all surviving pups from different litters, suggesting a partial effect on transplacental transmission due to a synergistic effect of the two antigens. Our results contrast with the 100% of vertical transmission previously described by vaccinating with rNcROP2Fam-1, despite the higher survival rates (Debache *et al.*, 2009). Conversely, no synergism was achieved by combining rNcGRA7 and rNcNTPase. Previous studies described a potentiated effect against cyst formation in *T. gondii* after vaccination with rTgROP+rTgROP4, which was not observed after single inoculation of these proteins regardless of the mouse strain employed (Dziadek *et al.*, 2012; Dziadek *et al.*, 2011; Dziadek *et al.*, 2009). In contrast, vaccination with rTgROP2, rTgGRA4 and rTgROP2+rTgGRA4 combination vaccine against *T. gondii* challenge infection in mice exhibited the same efficacy (Sanchez *et al.*, 2011), supporting our findings concerning the synergistic effect, but only in groups vaccinated with rhoptry proteins.

Morbidity scores after challenge in vaccinated mice ranged from 1 to 2, while the non-vaccinated and challenged mice reached the morbidity score 3. The low morbidity clearly reflected the very low rate of mortality in vaccinated mice. Previous studies employing the same animal model showed higher rates of morbidity and mortality, with the appearance of neurological symptoms, which led to death in some cases (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a). Hence, our results point towards a reduction of the severity of *N. caninum* infection in all vaccinated mice. Previous studies had also shown that vaccination of mice with rNcROP2Fam-1 protected against clinical signs of neosporosis (Debache *et al.*, 2010; Debache *et al.*, 2009; Debache *et al.*, 2008).

The effects of *N. caninum* infection in dams and non-pregnant mice are different, since the former are more susceptible to cerebral infection than the latter (Aguado-Martínez *et al.*, 2009a) due to the pregnancy-associated immune modulation (Athanassakis & Iconomidou, 1996). Dams vaccinated with rhoptry proteins exhibited the lowest cerebral parasite burden, while vaccination with dense granule proteins resulted in the highest cerebral parasite burden. In addition, the frequency of parasite detection in brains of dams vaccinated with rhoptry proteins was slightly lower than that observed in dams vaccinated with proteins from dense granules. Although these findings were not statistically significant, they could reflect a residual impediment of parasite replication due to vaccination with rhoptry proteins. IgG responses were also significantly higher in mice vaccinated with rNcGRA7 and rNcGRA7+rNcNTPase and lower in mice inoculated with rhoptry proteins after challenge. These results could be explained by means of the immunodominant role of NcGRA7, as mentioned above, or by a lower stimulation of the immune cells

in those groups immunized with in rhoptry proteins due to a lower number of replicating parasites. Nevertheless, several vaccine trials employing rNcSRS2 (Pinitkiatisakul *et al.*, 2007; Haldorson *et al.*, 2005; Pinitkiatisakul *et al.*, 2005), rNcPDI (Debache *et al.*, 2011b; Debache *et al.*, 2010), rNcMIC3 (Cannas *et al.*, 2003b), rNcROP2Fam-1 (Debache *et al.*, 2010; Debache *et al.*, 2008), chimeric protein rNcMIC3-MIC1-ROP2Fam-1 (Monney *et al.*, 2011) and bradyzoite proteins (rNcBAG1, rNcMAG1 and rNcSAG4; (Uchida *et al.*, 2013)) have evidenced the ability of these antigens to reduce the cerebral parasite load in infected mice. However, in terms of parasite burden, our results remained consistent with our previous studies (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a).

Since IFN-γ secretion has been associated with protective immunity against congenital transmission in mouse models (Long *et al.*, 1998), the cellular recall responses elicited by vaccination were analysed. Despite the predominance of IgG1, usually associated with a Th2-response (Liew, 2002), vaccination with rNcROP2Fam-1 induced the highest levels of IFN-γ secretion. Besides, the groups vaccinated with rNcROP40 and rNcROP40+rNcROP2Fam-1 secreted higher levels of IFN-γ than those vaccinated with proteins from dense granules. Therefore, the average expression of this cytokine was more pronounced in groups vaccinated with rhoptry proteins, which could explain the promising results obtained in terms of efficacy. However, the level of IFN-γ secretion did not correspond to the best results in terms of pup survival: splenocytes from the rNcROP2Fam-1 vaccinated group displayed the highest IFN-γ secretion, but only a 6.3% of pup survival was recorded for this group; in contrast, rNcROP40+rNcROP2Fam-1 vaccinated mice registered the highest pup survival rate (16.2%), but low levels of IFN-γ secretion in stimulated splenocytes. In addition, vaccination had a positive effect on median survival time, which increased in the majority of the groups regardless of the secreted levels of IFN-γ. Hence, these results strongly suggest that IFN-γ is not able to control parasite dissemination by itself as initially assumed (Kano *et al.*, 2005; Quinn *et al.*, 2004). However, confirmation of this hypothesis would require analysis of IFN-γ levels after challenge infection. Remarkably, IgG1 levels remained high during the induction and effector phases in most of the groups. These findings support the fact that humoral responses also play a key role in the protection against *N. caninum* infection, as previously demonstrated with both recombinant and live vaccines (Marugán-Hernández *et al.*, 2011b; Haldorson *et al.*, 2005). On the other hand, the highest IL-4 secretion was observed in splenocytes of the group vaccinated with rNcGRA7, according to previous results (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a), but the average secretion of this cytokine in all other groups was very low. Previous studies on TgROP2-vaccines against *T. gondii* showed high secretion of IFN-γ, according to our results, although IL-4 levels were also high (Dziadek *et al.*, 2012; Dziadek *et al.*, 2011; Sanchez *et al.*, 2011; Dziadek *et al.*, 2009). Paradoxically, high levels of IFN-γ were also detected when TgNTPase-II was employed as vaccine in a mouse model of toxoplasmosis (Tan *et al.*, 2011), whereas our results show the opposite picture.

Discrepancies in results from distinct studies in terms of efficacy in females and pups and immunological responses can be explained by several factors. First, three inbred strains of mice have been traditionally employed as experimental models of neosporosis, but differences in their responses after challenge have been demonstrated (Mols-Vorstermans *et al.*, 2013). Second, several studies have focused on non-pregnant mouse models (Monney & Hemphill, 2014;

Hemphill *et al.*, 2013), for which the regulation of immunity is obviously not equal to those found during pregnancy. Third, the BALB/c model employed here ensures a 100% congenital transmission and 0% of survival of the offspring in the non-vaccinated mice (Dellarupe *et al.*, 2014a; López-Pérez *et al.*, 2008), which is highly dependent of the type of isolate used for challenge, since differences in isolate virulence have been demonstrated (Regidor-Cerrillo *et al.*, 2010a; Collantes-Fernández *et al.*, 2006b; Atkinson *et al.*, 1999). Fourth, direct comparisons with studies carried out in mouse models for *T. gondii* infection must be carefully considered despite the common phylogenetic origin of *T. gondii* and *N. caninum*, since relevant genetic dissimilarities that affect their infection biology have been recently elucidated (Reid *et al.*, 2012). The present study takes into account the worst scenario of neosporosis. Hence, despite the seemingly low level of protection achieved herein, our results are encouraging for the development of future approaches. In addition, the obvious discrepancies between our results and previous studies highlight the urgent need to adopt a common mouse model that allows a valid comparison of results from different research groups.

5. Conclusions

This is the first study that employs NcROP40 and NcNTPase as vaccines against *N. caninum* in a well-established pregnant mouse model. A combination vaccine consisting of rNcROP40 and rNcROP2Fam-1 achieved a synergistic effect, and resulted in a partial block of transplacental transmission. Therefore, these results suggest a potential for the use of rhoptry proteins as antigens in vaccine formulations as a novel strategy to block the processes in which they are involved. In contrast to vaccination with rhoptry proteins, vaccination with rNcGRA7 and rNcNTPase, alone or in combination, did not prevent congenital transmission and pup mortality after challenge despite an increased median survival time. Future investigations are required to establish the functional relevance of ROP2 family proteins in host-cell modulation and virulence, since no *N. caninum* virulence factors have been identified to date.

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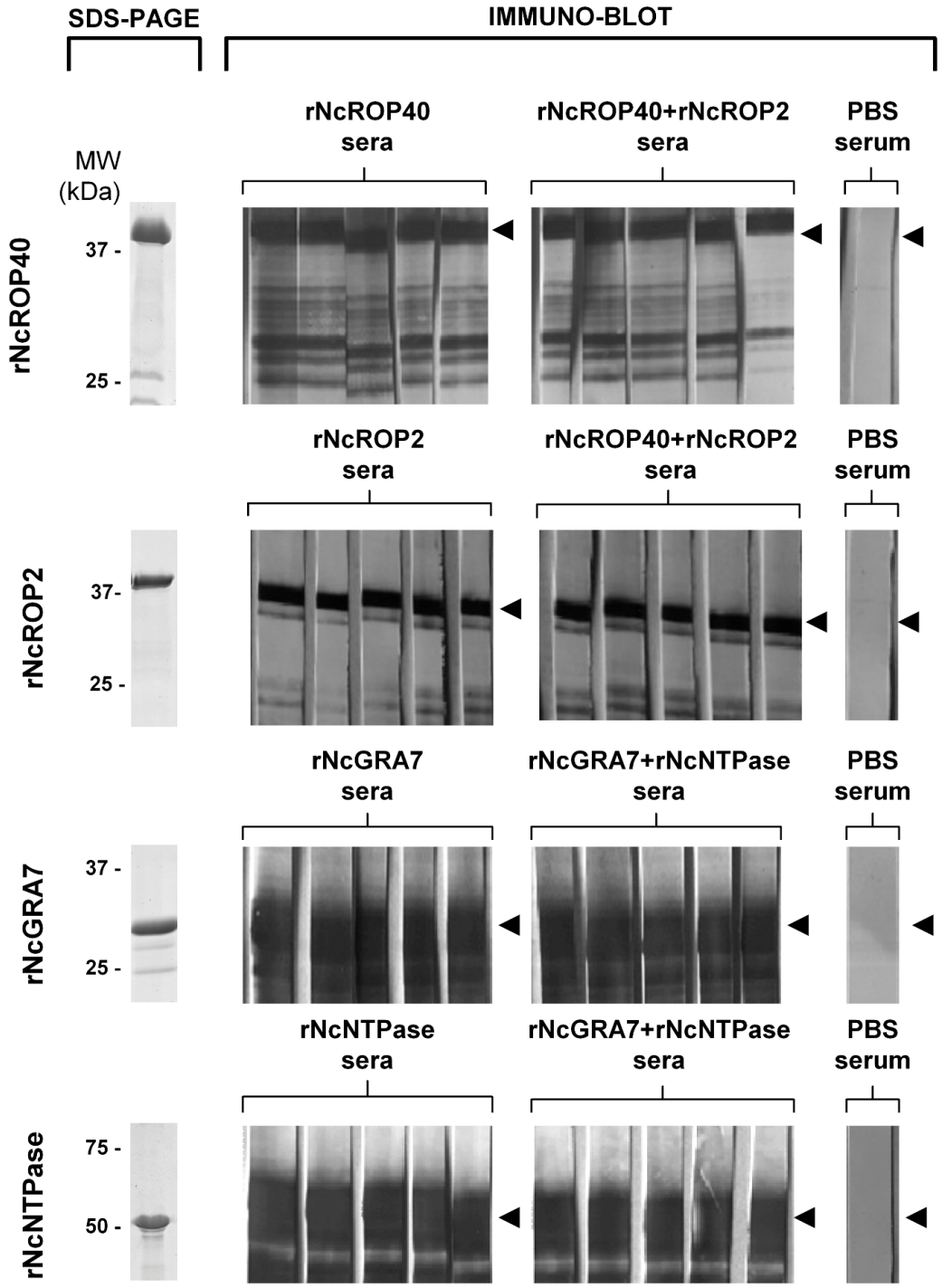
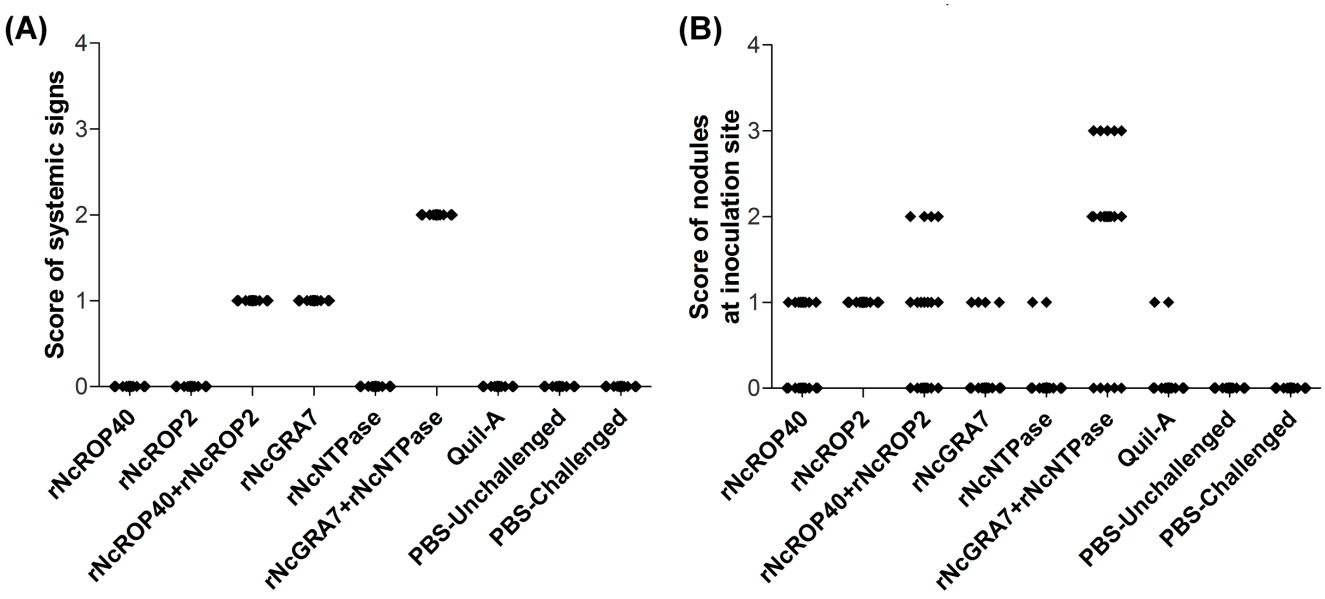
Author contributions

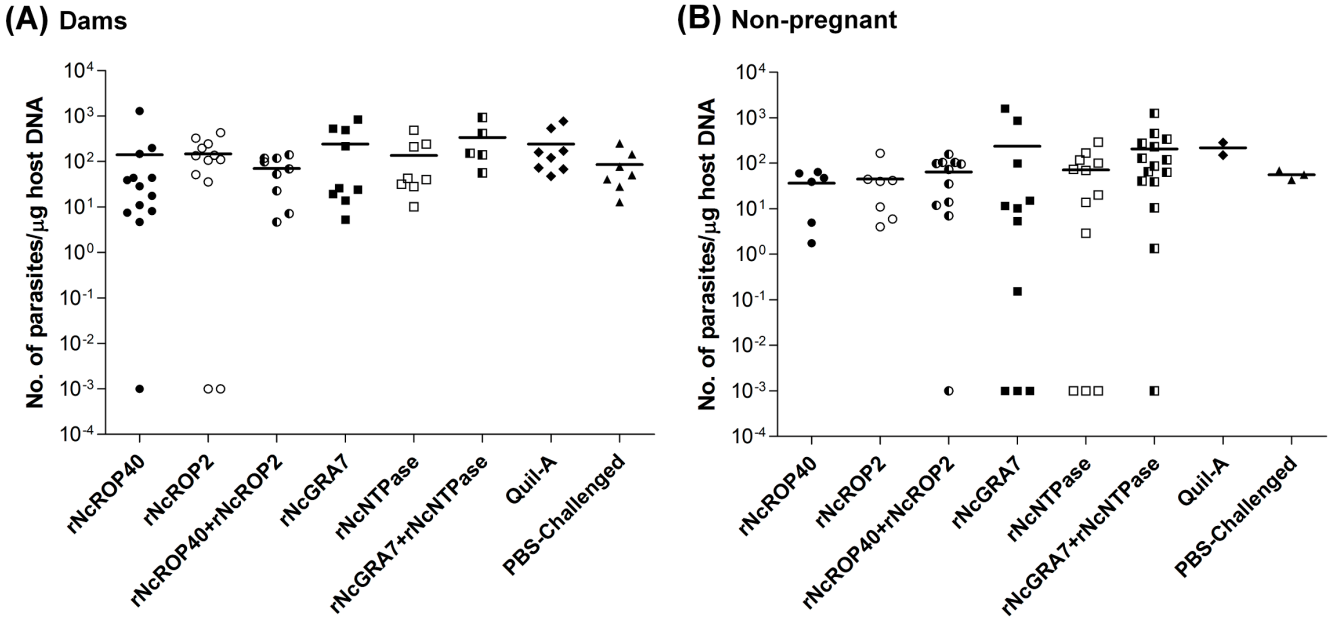
JRC, GAG and LMOM conceived and designed the experiments. IPF, DAS, AGC, MCCM and AH performed the experiments. IPF, JRC, GAG and LMOM analyzed the data. IPF, JRC, GAG, AH and LMOM wrote the paper



Supplementary Figure 1: Immunogenicity of vaccine antigens. Recombinant proteins NcROP40, NcROP2Fam-1, NcGRA7 and NcNTPase were separated by SDS-PAGE, stained with Coomassie blue (left column) and their immunogenicity were checked (right column). Sera from vaccinated mice (indicated on the top) were analyzed by Western-Blot against each recombinant protein. Head arrows on the right of immunoblots mark the presence of the recombinant protein on the nitrocellulose membrane and its specific recognition by mice in each case. Molecular weight pattern is indicated on the left of each row.



Supplementary Figure 2: Safety scores of inoculated formulations. Safety of vaccines was based on the presence of systemic side effects after vaccination (A) and the appearance of nodules at the inoculation site (B). The meaning of each score is summarized in Table 2. Symbols (◆) indicate single animals.



Supplementary Figure 3: Parasite burden in brains. Scatter plot representing the individual burden values (dots) and their mean (lines) in dams (panel A) and non-pregnant mice (panel B) at day 30 post-partum. No differences were found among groups ($P > 0.05$, Kruskal-Wallis *H* test).

Supplementary table 1: Primers used to amplify Nc*ROP40*, Nc*ROP2Fam-1*, Nc*GRA7* and Nc*NTPase* sequences

Protein	Primer sequences	Length	Annealing temperature
rNcROP40	Fw-*Sac*I-CGAGCTCATGGTGAAATCCCTGCACAAG Rv-*Pac*I-CCTTAATTAATCACCCCACCACTGAACGC	1176 bp. 1-392 aa.	60º C
rNcROP2Fam-1	Fw-*Sac*I-GAGCTCTTGTGGCGTAATCAGAAGCAC Rv-*Hind*III-AAGCTTTTATAGCCTCGTGTCCTCCGT	1076 bp. 236-595 aa.	58º C
rNcGRA7	Fw-*Bam*HI-TAATGGATCCGAGGAGACTTGGCAACCGAA Rv-*Eco*RI-TTGGAATTCCCTATTCGGTGTCTACTTCC	572 bp 28-217 aa.	56º C
rNcNTPase	Fw-*Sac*I-GAGCTCATGGCCGACGAGCCAGCGACACTT Rv-*Pac*I- GGCCTTAATTAATCACATGTTGTAGGTAAATCCCG	1808 bp 24-627 aa.	56º C (x10 cycles) 62º C (x25 cycles)

GENERAL DISCUSSION

Neospora caninum is considered to be a major cause of abortion in cattle worldwide, being responsible for important economic losses exceeding one billion dollars per annum (Reichel *et al.*, 2013). However, vaccines or drugs against bovine neosporosis are not yet available. In this scenario, vaccination has proven to be an economically viable strategy for controlling the disease (Reichel *et al.*, 2013), and control options are reduced to interrupting the parasite life cycle through management measures (Reichel *et al.*, 2014). Hence, the development of effective vaccines against the disease emerges as an urgent need. Despite live vaccines having demonstrated high efficacy rates, their use entail several disadvantages such as their potential for reversion to pathogenicity and costly production (Reichel *et al.*, 2015). In this sense, subunit vaccines appear as a good alternative to the use of live vaccines due to their safety and reduced costs. In addition, they can be specifically designed against particular proteins to block essential processes for the parasite survival (Monney & Hemphill, 2014). Unfortunately, the basic mechanisms that govern cell invasion and proliferation by *N. caninum* tachyzoites are still largely unknown at the molecular level. The occurrence of variations in genetic profiles, *in vitro* behaviour, and pathogenicity among *N. caninum* isolates have all been shown, but the identification of potential virulence factors has not been exploited. Thus, the choice of proper targets to design new vaccine formulations against neosporosis is a difficult task (Hemphill *et al.*, 2013).

In this context, the present doctoral thesis covers three main objectives: *i*) the determination of the proteome and immunome changes among two virulent and one non-virulent *N. caninum* isolates in order to identify proteins potentially related to parasite virulence; *ii*) the characterization of those identified proteins exclusively found in apicomplexan parasites from secretory organelles and scarcely investigated to date; and *iii*) the evaluation of the usefulness of such proteins formulated as subunit vaccines against neosporosis in mouse models.

The first objective aimed to compare the protein and antigenic composition of different *N. caninum* isolates by two complementary proteomic approaches. Several works have previously proven the influence of *N. caninum* isolate in the outcome of the infection (Dellarupe *et al.*, 2014a; Dellarupe *et al.*, 2014b; Regidor-Cerrillo *et al.*, 2011; Regidor-Cerrillo *et al.*, 2010a; Rojo-Montejo *et al.*, 2009a; Rojo-Montejo *et al.*, 2009b). However, the elements underlying this pathogenic diversity remain largely unresolved. Consequently, DIGE and MALDI TOF-MS analyses were used to investigate the potential differences in the proteome among well-characterized virulent (Nc-Liv and Nc-Spain7) and non-virulent (Nc-Spain1H) isolates. On the other hand, an additional approach was conducted to further investigate the potential influence of the same isolates on the immune response pattern elicited from experimentally infected mice by 2-DE immunoblotting coupled to MS/MS.

Nineteen protein spots were identified by MALDI TOF-MS, corresponding to 11 proteins classified into four main groups related to parasite motility (NcACT1, and NcMLC1), components of the secretory organelles (NcMIC1, NcROP9, NcROP40, and NcNTPase), parasite metabolism (glucose-6-phosphate

dehydrogenase [G6PD], and 6-phosphogluconate dehydrogenase [6PGD]), and miscellaneous functions (voltage-dependent anion channel and aspartyl-tRNA synthetase). We hypothesize that the identification of differentially abundant or recognized proteins among virulent and non-virulent isolates would be a valuable tool for obtaining new vaccine candidates against bovine neosporosis. Previous studies carried out with uncharacterized isolates by 2-DE approaches failed to connect tachyzoite proteome changes with parasite virulence (Lee *et al.*, 2005; Shin *et al.*, 2005b). In contrast, differences in expression of TgACT1, TgSAG1, TgMIC5, TgGRA1-8, TgNTPase 3 and catalase have been associated with the virulence of different Type I *T. gondii* isolates (Choi *et al.*, 2010; Nischik *et al.*, 2001).

Despite the Nc-Liv, Nc-Spain7 and Nc-Spain1H isolates showing marked differences in protein abundance, immunome analyses did not find substantial variations in their antigenic profile under the same pooled sera. Hence, no specific antigens were found for any isolate. In previous studies, the KBA2 and JPA1 isolates exhibited almost identical 2-DE patterns when their proteomes and immunomes were compared by employing rabbit anti-*N. caninum* KBA2 serum (Lee *et al.*, 2005). Shin *et al.* (2005) also described similar 2-DE proteomes and immunomes between KBA2 and VMDL-1 isolates using bovine anti-*N. caninum* KBA2 serum. Herein, the major differences observed in the immunome pattern uniquely depended on the pool of sera used regardless of the isolate protein extract employed. Sera from mice experimentally infected with Nc-Liv showed the most complete pattern of antigen recognition, whilst sera from mice experimentally infected with Nc-Spain1H achieved the lowest number of spots detected. A total of 17 immunogenic spots were identified by MS/MS, and were classified into three main groups related with parasite motility (NcACT1, NcMLC1, NcTUB-β, and NcGAP45), components of the secretory organelles (NcROP1, serine-threonine phosphatase 2C, NcSUB1, NcGRA1, NcGRA2, and NcGRA7), and oxidative stress and metabolism (HSP70, superoxide dismutase, and fructose-1,6-bisphosphate aldolase). Remarkably, Nc-Spain1H sera failed to detect four previously identified proteins (GAP45, serine-threonine phosphatase 2C, NcGRA1, and superoxide dismutase), and three additional spots and spot chains recognized by Nc-Liv and Nc-Spain7 sera, but not successfully identified. These variations could be due to the differences in the infection dynamics among *N. caninum* isolates (Jiménez-Ruiz *et al.*, 2013a; Pereira García-Melo *et al.*, 2010), and suggest a hierarchical pattern of antigen recognition. This antigen recognition would imply that the immunodominant antigens are firstly recognized by the host immune system, followed by other minor antigens during the course of the infection. In accordance with this hypothesis, mice infected with the Nc-Liv and Nc-Spain7 isolates showed higher antibody levels compared with Nc-Spain1H infected mice. In this sense, a previous study demonstrated that isolate virulence was clearly correlated with higher IgG levels against NcGRA7 antigen (Jiménez-Ruiz *et al.*, 2013b). Therefore, the lack of recognition of specific spots by sera from mice infected with non-virulent isolates suggests that the control of the Nc-Spain1H-mediated infection does not rely on specific immune responses against isolate-specific antigens.

In summary, these proteomic approaches have demonstrated he occurrence of clear differences in the protein abundance and the elicited immune response by the three different *N. caninum* isolates

studied. All the identified proteins were associated with relevant processes of the lytic cycle such as gliding motility, secretion of protein effectors into the host cell, and stress responses to prevent the intracellular killing of the tachyzoites after invasion.

The NcACT1 and NcMLC1 proteins are differentially expressed among isolates. In addition, the NcACT1, NcMLC1, NcTUB-β, and NcGAP45 proteins showed to be immunogenic antigens, although recognition of the NcGAP45 protein was restricted to sera from mice experimentally infected with the Nc-Liv isolate. Actin, myosin, and tubulin integrate the glideosome, the microtubules and the apical conoid, and are related to parasite gliding motility. In *T. gondii* gliding motility is essential for the lytic cycle success, and it is mediated by an actin/myosin-based motor complex that is anchored to the inner membrane complex. This motor complex comprises the interaction of the TgACT1, TgMLC1, TgMyoA, TgGAP45, and TgGAP50 proteins, and controls parasite motility, invasion and replication (Egarter *et al.*, 2014; Mehta & Sibley, 2011; Heaslip *et al.*, 2010; Agop-Nersesian *et al.*, 2009; Daher & Soldati-Favre, 2009; Gilk *et al.*, 2009; Carruthers & Boothroyd, 2007; Sahoo *et al.*, 2006). Consequently, NcACT1, NcMLC1 and NcGAP45 proteins could play similar roles in *N. caninum* and determine an increased invasion capacity as demonstrated in the high virulence isolates (Regidor-Cerrillo *et al.*, 2011).

The NcMIC1 protein is more abundant in *N. caninum* virulent isolates, and is secreted immediately after parasite adhesion to the host cell to stabilize the interaction between tachyzoite and host cell during invasion (Keller *et al.*, 2002). In *T. gondii*, the invasion success is dependent on the association of TgMIC1, TgMIC4 and TgMIC6 proteins. Indeed, deletion of both TgMIC1 and TgMIC3 leads to a decrease in the parasite virulence (Cerede *et al.*, 2005). Thus, the decreased abundance of NcMIC1 in Nc-Spain1H isolate may be associated with its reduced invasiveness and pathogenicity (Regidor-Cerrillo *et al.*, 2011; Rojo-Montejo *et al.*, 2009b).

Rhoptry proteins are implicated in the creation of the moving junction, the development of the nascent parasitophorous vacuole, and the regulation of host cell processes. However, the functionality for these proteins is unknown in *N. caninum* and limited in *T. gondii* (Qiu *et al.*, 2009; Boothroyd & Dubremetz, 2008; Reichmann *et al.*, 2002). Within the present objective, the NcROP40 pseudokinase showed to be more abundant in the high virulence isolates, whereas the rhoptry protein NcROP1 was identified as an immunogenic antigen. These proteins had been previously identified in subcellular fractions enriched for rhoptry elements by LC/MS-MS (Marugán-Hernández *et al.*, 2011a), and may represent key effectors for the parasite virulence. On the other hand, the serine-threonine phosphatase 2C was consistently detected in 2-DE immunoblots by Nc-Liv and Nc-Spain7 sera. Phosphatases are highly conserved in apicomplexan parasites and regulate a wide variety of cellular functions (Li *et al.*, 2012). For instance, the *T. gondii* protein phosphatase 2C, which is stored in the rhoptries, is secreted and targeted to the host nucleus during invasion (Gilbert *et al.*, 2007). To date, the specific function of the serine-threonine phosphatase 2C has not been clarified, but according to our results, the protein may be relevant for the strain virulence.

Furthermore, the dense granules protein NcNTPase was more abundant in the Nc-Spain7 and Nc-Liv isolates. This protein has enzymatic activity, is secreted during the host-cell invasion and presents multiple gene copies (Asai *et al.*, 1998). Two different NTPase isoforms have been identified in *T. gondii*, and one of them has been associated with parasite virulence (Asai *et al.*, 1995). Therefore, NcNTPase activity may be necessary for the survival and proliferation of the parasite during intracellular development as suggested for *T. gondii* (Asai *et al.*, 2002; Nakaar *et al.*, 1999). Besides, the NcGRA1, NcGRA2 and NcGRA7 proteins were also identified as immunogenic antigens. Interestingly, sera from Nc-Spain1H-infected mice failed to recognize the NcGRA1 protein. NcGRA1, NcGRA2 and NcGRA7 are secreted into the PV during and after invasion, indicating that they are required for its maturation (Aguado-Martínez *et al.*, 2010; Vonlaufen *et al.*, 2004; Atkinson *et al.*, 2001). Therefore, PV development and the subsequent replication could be more efficiently accomplished by highly virulent isolates. With regard to NcGRA7, this protein has been described as a major IDA for bovine neosporosis (Álvarez-García *et al.*, 2007), proposed as an abortion marker in pregnant cattle and associated with the isolate virulence in mice (Jiménez-Ruiz *et al.*, 2013b; Huang *et al.*, 2007).

G6PD and 6PGD enzymes were more abundant in the Nc-Spain7 protein extract. In *T. gondii*, both proteins operate in the oxidative branch of the pentose phosphate pathway (PPP) to generate NADPH and protect the parasite from oxidative stress caused by NO delivered during the Th1-type immune response (Pinheiro *et al.*, 2010). Hence, these proteins may be responsible for high *in vitro* tachyzoite yield and pathogenicity showed by Nc-Spain7 and Nc-Liv isolates (Regidor-Cerrillo *et al.*, 2011; Regidor-Cerrillo *et al.*, 2010a; Gibney *et al.*, 2008; Williams *et al.*, 2000). In the same way, superoxide dismutase failed to be consistently detected in 2-DE immunoblots by Nc-Spain1H sera. This enzyme helps to protect *T. gondii* and *N. caninum* from oxygen toxicity, and might play an important role protecting highly virulent isolates from oxidative killing by host effector cells (Cho *et al.*, 2004).

Expression differences among isolates were also detected in a porin family member with predicted voltage-dependent anion channel (VDAC) activity, whose homologues participate in the transport of metabolites across the mitochondrial outer membrane, including the ATP/ADP exchange (Peixoto *et al.*, 2010; Rostovtseva & Bezrukov, 2008). Finally, an aspartyl-tRNA synthetase (AspRS)-like protein was over-expressed in the Nc-Spain7 isolate. Aminoacyl-tRNA synthetases are ubiquitous enzymes essential for protein synthesis, RNA splicing and trafficking, and apoptosis (Bour *et al.*, 2009; Park *et al.*, 2005).

SRS family comprises immunodominant antigens that are attached to the outer membrane of the parasite and mediate host cell attachment and invasion (Jung *et al.*, 2004; Howe & Sibley, 1999). Besides, proteins from the *T. gondii* ROP2-kinase family have shown to be relevant virulence factors that are secreted into the host cell and interact with the PVM (Boothroyd & Dubremetz, 2008; El Hajj *et al.*, 2006). Therefore, variations in the abundance of these proteins may also contribute to phenotypic differences

displayed by these three *N. caninum* isolates. However, these proteins were scarcely identified by our proteomic approaches. This is likely due to the hydrophobic nature of membrane proteins, which impedes their proper resolution by 2-DE techniques, and the lower abundance of secreted proteins. Hence, large-scale experiments including a higher number of *N. caninum* isolates and complementary proteomic platforms could help to identify new elements implicated in the parasite pathogenicity.

In summary, proteomic approaches have shown to be useful to determine potential variations in the tachyzoite proteome and immunome from different *N. caninum* isolates. On one hand, the consistent variations in NcMIC1, NcROP40, NcNTPase, G6PD and aspartyl-tRNA synthetase abundance among virulent and non-virulent isolates may determine the differences in their *in vitro* and *in vivo* behaviour (Dellarupe *et al.*, 2014a; Dellarupe *et al.*, 2014b; Regidor-Cerrillo *et al.*, 2011; Regidor-Cerrillo *et al.*, 2010a; Rojo-Montejo *et al.*, 2009a; Rojo-Montejo *et al.*, 2009b). On the other hand, the lack of recognition of the NcGAP45, serine-threonine phosphatase 2C, superoxide dismutase, and NcGRA1 proteins by the Nc-Spain1H sera suggests their potential use as virulence markers. Despite *N. caninum* IDAs having shown to be highly conserved among natural and experimentally infected hosts (Lee *et al.*, 2005; Shin *et al.*, 2005b; Shin *et al.*, 2004; Lee *et al.*, 2003; Álvarez-García *et al.*, 2002; Bjerkås *et al.*, 1994), the usefulness of the above mentioned markers should be further investigated by employing cattle sera.

The second objective of the present doctoral thesis is aimed at characterizing, for the first time, those proteins potentially involved in parasite virulence. We hypothesize that differences in protein abundance or immune recognition among isolates may play a key role in the phenotypic differences between them and influence the immune recognition by the host. Consequently, those proteins or antigens differentially identified would be further characterized. The characterization studies were focused on the NcROP40 and NcNTPase proteins according to these criteria: *i*) both proteins are more abundant in virulent isolates; *ii*) they are found in secretory organelles exclusively present in apicomplexan parasites; and *iii*) the current information about both proteins is quite limited. More specifically, NcROP40 belongs to the ROP2 family of protein kinases, which include *T. gondii* virulence factors. In fact, NcROP2Fam-1 is the only rhoptry protein characterized to date in *N. caninum*, and has shown high efficacy rates when tested as subunit vaccine in mice (Alaeddine *et al.*, 2013; Debache *et al.*, 2008). Similarly, the TgNTPase protein has been also suggested as a virulence factor of *T. gondii* (Asai *et al.*, 1995). In contrast, the knowledge about NcMIC1 and NcGRA1 has been extensively exploited (Hemphill *et al.*, 2013).

To date, a number of rhoptry proteins, including NcROP40, have been identified in *N. caninum* (Regidor-Cerrillo *et al.*, 2012; Marugán-Hernández *et al.*, 2011a; Sohn *et al.*, 2011; Straub *et al.*, 2009). However, ROP40 function has not been determined either in *N. caninum* or in *T. gondii* despite the fact that orthologous Tg*ROP40* (Tg*ROP2L6*) seems to be necessary for the acute stage of the disease (Pittman *et al.*, 2014; Peixoto *et al.*, 2010). On the other hand, the available data for the NcNTPase protein is restricted to their enzymatic activity and crystallographic structure (Matoba *et al.*, 2010; Asai *et al.*, 1998). NTPases (or

NTPDases) are widely extended among eukaryotes (Sansom, 2012), but they seem especially relevant for the members of the Sarcocystidae family (Zhang *et al.*, 2006; Asai *et al.*, 1998; Asai *et al.*, 1995). In *T. gondii* some NTPase isoforms are restricted to the virulent strains (Asai *et al.*, 1995), which is consistent with our DIGE findings. Nevertheless, there is no consensus regarding their specific function, which has been associated with the purine salvage, the tachyzoite proliferation and egress, and the suppression of the host immune responses (Tonin *et al.*, 2013; Sansom, 2012; Melo *et al.*, 2011; Nakaar *et al.*, 1999; Silverman *et al.*, 1998; Sibley *et al.*, 1994).

In the present objective, NcROP40 and NcNTPase characterizations were addressed through an exhaustive analysis of their genomic sequences, protein features, and subcellular localization. Moreover, both proteins were compared with the previously characterized NcROP2Fam-1 and NcGRA7, respectively (Alaeddine *et al.*, 2013; Aguado-Martínez *et al.*, 2010), and analysed in the context of the host-parasite relationships through a descriptive approach that included the study of the protein dynamics, secretion, phosphorylation, and transcript profile throughout the tachyzoite lytic cycle.

With regard to NcROP40, the protein is localized in rhoptry bulbs. Western-blot analyses showed that NcROP40 is apparently modified through a proteolytic process within the parasite. This proteolytic maturation has been previously shown in many ROP proteins, both in *T. gondii* and *N. caninum* (Alaeddine *et al.*, 2013; Hajagos *et al.*, 2012). In *T. gondii* TgSUB2 protease is in charge to remove the N-terminal domains that are involved in rhoptry targeting at a highly conserved SΦx(E/D) site (Hajagos *et al.*, 2012). This sequence was also found in the N-terminal domain of NcROP2Fam-1 (Alaeddine *et al.*, 2013), but is absent in NcROP40. Hence, it remains unclear whether this protein is processed by different proteases in *N. caninum*. Moreover, a number of studies suggest that NcROP40 is one of the major components present in the tachyzoite rhoptries, since it has been consistently identified by three different proteomic approaches (Pollo-Oliveira *et al.*, 2013; Regidor-Cerrillo *et al.*, 2012; Marugán-Hernández *et al.*, 2011a).

Nc*ROP40* sequence analyses showed the lack of an annotated N-terminus on the protein ORF, and lead to the description of the Nc*ROP40*-long sequence, a corrected annotation of the Nc*ROP40* gene including an additional 402 bp in its N-terminal fragment. These results were confirmed by RT-PCR, and were in accordance with an improved transcriptome annotation for NcROP40 (Ramaprasad *et al.*, 2015). In addition, Western-blot analyses corroborated the existence of the additional N-terminus on the protein sequence. On the other hand, the NcROP40 protein has been recently established as a predicted member of the rhoptry kinase family, lacking the key kinase sequence motifs present in the C-terminus (Talevich & Kannan, 2013). Comparison of the Nc*ROP40* ORF among Nc-Liv, Nc-Spain7 and Nc-Spain1H isolates did not reveal any polymorphism that could explain differences in virulence as described for TgROP18 (Steinfeldt *et al.*, 2010). Nevertheless, variations in protein abundance among isolates might be due to regulatory mechanisms such as epigenetics, which have been shown to be involved in genome reprogramming during tachyzoite to bradyzoite conversion in *T. gondii* (Dixon *et al.*, 2010).

NcROP40 includes a signal peptide in its sequence, whose presence is an important pre-requisite for a protein to enter the secretory pathway. However, no evidence for NcROP40 secretion was observed by confocal microscopy. Moreover, NcROP40 does not interact with the PVM, albeit this finding is consistent with the predicted lack of RAH domains in its sequence. RAH domains are also absent in the TgROP40 protein, and consequently it does not associate with the PVM (Reese & Boothroyd, 2009; El Hajj *et al.*, 2006). In this sense, previous studies carried out with the *T. gondii* rhoptry protein toxofilin showed that secretion of low abundance proteins may be undetectable by these approaches, which is especially relevant for those proteins that are not concentrated on a membrane or in an intracellular compartment (Lodoen *et al.*, 2010). This may be the case for the NcROP40 protein that could be secreted into the host cell cytosol and translocated to the host nucleus as previously reported for the TgROP16 protein (Saeij *et al.*, 2007). Accordingly, the orthologous TgROP40 protein showed some nuclear localization when it was heterologously expressed in infected HFF, which supports this hypothesis and suggests a potential role of the protein on host cell regulation (Reese & Boothroyd, 2009). In contrast to NcROP40, NcROP2Fam-1 is detected in evacuoles as described earlier (Alaeddine *et al.*, 2013), and then surrounding invasive tachyzoites from 1 to 24 hpi. Similar findings have been described for the TgROP2 protein, which may participate in the PVM maturation (Dunn *et al.*, 2008; Nakaar *et al.*, 2003; Sinai & Joiner, 2001; Beckers *et al.*, 1994). Hence, a similar function could be expected for NcROP2Fam-1. Remarkably, we could not detect any NcROP40 and NcROP2Fam-1 protein in the secretory fractions after induction of tachyzoite secretion using A23187, ethanol or DTT stimulation. This indicates that rhoptry discharge is not affected by elevated intracellular calcium levels as previously stated for *T. gondii*, and its secretion may be only induced upon host cell contact (Carruthers & Sibley, 1999). All together, these experiments demonstrate different features of both proteins.

Herein, we established the NcNTPase as a canonical GRA protein according to the criteria described earlier (Mercier & Cesbron-Delauw, 2015). Besides this, we describe the presence of three different *loci* for the Nc*NTPase* gene within the Nc-Liv genome. Our sequence analyses showed that Nc*NTPase 2* lacks the up- and down-stream elements shared by Nc*NTPase 1* and *3*. Interestingly, *T. gondii* only expresses the Tg*NTPase 1* and *3*, which is under the control of an active promoter solely present in those alleles (Nakaar *et al.*, 1998; Asai *et al.*, 1995). This is indicative of a similar promotor structure for the Nc*NTPase* alleles. In addition, we identified up to six different Nc*NTPase* alleles. Previous studies in *T. gondii* reported the presence of three tandemly repeated copies of the Tg*NTPase* gene (Bermudes *et al.*, 1994). Asai and colleagues then also detected up to three different alleles of the Nc*NTPase* gene by Southern-blot analyses in *N. caninum* (Asai *et al.*, 1998). In the present work we also demonstrate the presence of diverse protein species of the NcNTPase in the Nc-Liv isolate, which is in accordance with previous studies (Lee *et al.*, 2003; Shin *et al.*, 2005; Regidor-Cerrillo *et al.*, 2012). Therefore, it is likely that *N. caninum* expresses different isoforms of the NcNTPase protein as reported earlier for *T. gondii* (Kikuchi *et al.*, 2001). According to this, further studies should be carried out in order to clarify the impact of the NcNTPase expression on parasite pathogenicity. However, the Nc*NTPase* 2 and 3 copies show a reading frame shift caused by a

single-nucleotide insertion, and thus, it is unlikely that both genes are properly translated. Remarkably, it has been recently described that gene duplications are notoriously underestimated due to collapsing of the assembly in regions containing tandemly duplicated clusters of similar genes (Adomako-Ankomah *et al.*, 2014). More specifically, Adomako-Ankomah and colleagues have shown the existence of expanded *loci* carrying a variable number of alleles coding for the TgNTPase protein, the numbers of which depend on the isolate. These findings support our evidence on the wide variety of transcribed Nc*NTPase* alleles that we have observed, which could be harboured within the Nc*NTPase loci*. Consistent with this, we failed to amplify the whole Nc*NTPase 1* gene (coding sequence and flaking regions) by PCR, even when employing specific long-range PCR methods. In this scenario, the availability of different genome annotations from diverse *N. caninum* isolates would be highly desirable, but to date only the Nc-Liv genome has been fully sequenced (Reid *et al.*, 2012).

Additionally, we performed a detailed tracing of the NcNTPase and NcGRA7 proteins throughout the lytic cycle. NcNTPase and NcGRA7 secretion was detected during early invasion, PV maturation, and egress. Besides, both proteins exhibited a clear interaction with the PVM, as previously reported for the TgNTPase (Bermudes *et al.*, 1994) and the NcGRA7 proteins (Aguado-Martínez *et al.*, 2010). In contrast, as shown by the co-localisation studies, the temporal distribution of the NcNTPase and NcGRA7 proteins were distinct during invasion and egress, suggesting differences in the protein trafficking at these stages. Furthermore, we found that NcNTPase secretion occurs in tachyzoites undergoing egress regardless of the applied treatment (no treatment, A23187, ethanol, or DTT). Similar findings have been previously reported for the SnNTPase protein from *S. neurona*, which can be detected in secreted fractions after incubation at 37 ºC without further treatment (Zhang *et al.*, 2006). The NcGRA7 protein displayed a similar behaviour, although secretion at 4º C was apparently less pronounced. Consistent with this, it has been showed that *T. gondii* GRA proteins are constitutively released in a calcium-independent and a temperature-dependent fashion (Phelps *et al.*, 2008; Carruthers & Sibley, 1999; Chaturvedi *et al.*, 1999). However, our results suggest that NcNTPase discharge is under a distinct regulation mechanism. In contrast to dense granules, microneme secretion can be specifically induced by calcium ionophores and DTT both in *T. gondii* and *N. caninum* (Carruthers & Sibley, 1999; Naguleswaran *et al.*, 2001; Pastor-Fernández *et al.*, 2015b). According to this, all the applied treatments specifically induced NcMIC2 secretion as described earlier (Lovett *et al.*, 2000; Pastor-Fernández *et al.*, 2015b), whereas they had no effect on NcNTPase secretion.

NcROP40, NcROP2Fam-1, NcNTPase and NcGRA7 transcripts displayed the highest mRNA levels at 6 and 56 hpi, coinciding with egress and/or early invasion. Hence, we hypothesize that their function may be necessary to guarantee the lytic cycle progression, highlighting the relevance of rhoptry and dense granules proteins for the invasion and proliferation processes in *N. caninum*. This phenomenon is consistent with the "just-in-time" concept stated for *P. falciparum* and *T. gondii*, whereby genes are only activated as their biological function becomes necessary to the parasite (Behnke *et al.*, 2010; Radke *et al.*,

2005; Llinas & DeRisi, 2004). Consistent with this, a modal switch from expression of proteins involved in invasion and motility of *T. gondii* has been also described in egressed tachyzoites (Gaji *et al.*, 2011; Lescault *et al.*, 2010).

On the other hand, DTT treatment induced a dramatic increase in NcROP2Fam-1, NcGRA7 and NcNTPase expression to levels similar to naturally occurring egress. Strikingly, NcROP40 expression was not substantially increased by this treatment, suggesting a different transcriptional regulation for this gene. These findings also suggest that *i*) NcROP2Fam-1, NcGRA7 and NcNTPase could have a dual role during egress and invasion, and *ii*), NcROP40 may be only essential during or after early invasion. Interestingly, *T. gondii* secretes the reducing agent glutaredoxin to activate the TgNTPase as replication increases, supporting our hypothesis regarding the crucial role of the protein function during egress. Indeed, DTT has shown to activate the TgNTPase protein *in vitro* and to trigger the egress (Krug *et al.*, 2013; Stommel *et al.*, 2001). Although the processes governing the egress are not fully understood, intracellular calcium levels seems to trigger the abrupt exit of parasites from PV, which is accompanied with a rapid decrease in host cell ATP mediated by the TgNTPase activation (Blackman & Carruthers, 2013). Hence, we hypothesize that egress regulation in *N. caninum* relies on NcNTPase activation, which is accompanied by an increase in the expression of secreted effectors (NcGRA7 and NcROP2Fam-1) necessary to accomplish new invasion waves in the neighbouring cells (Pastor-Fernández *et al.*, 2015b). In fact, TgNTPase has been previously suggested to act as a timer for the *T. gondii* lytic cycle (Santos *et al.*, 2009). Since egress and invasion represent critical processes in which parasites are highly exposed to the host immune system, NcNTPase secretion could counteract the development of inflammatory responses to avoid tachyzoite clearance. Interestingly, it has been suggested that the TgNTPase may suppress the local host immune responses (Tonin *et al.*, 2013; Sansom, 2012; Melo *et al.*, 2011; Santos *et al.*, 2009), supporting this hypothesis.

Finally, phosphorylation of NcROP40, NcROP2Fam-1, NcNTPase and NcGRA7 was investigated due to its prominent role in cellular regulatory processes and its influence on the functional activity of several proteins. Intriguingly, protein phosphorylation of NcROP2Fam-1, NcGRA7 and NcNTPase was evident during egress, and coincided with the maximum transcript levels and with the protein release into the extracellular environment. These observations might indicate a common regulation mechanism necessary for their participation within the lytic cycle. In contrast, NcROP40 phosphorylation was not observed at this point. Previous works have indicated that the TgROP2, TgROP4 and TgGRA7 proteins are solely phosphorylated in intracellular parasites (Dunn *et al.*, 2008; Carey *et al.*, 2004; Neudeck *et al.*, 2002). Interestingly, phosphorylation of TgGRA6 has been shown to coincide with its association with the PVM (Mercier *et al.*, 2005; Labruyere *et al.*, 1999), and this could also be the case for NcNTPase and NcGRA7 proteins, which are also localized at the periphery of the PV. Nevertheless TgGRA7 was shown to associate with the PVM independently of its phosphorylation status (Dunn *et al.*, 2008; Coppens *et al.*, 2006; Neudeck *et al.*, 2002). In fact, it was suggested that TgGRA7 modification might regulate the formation of complexes

with other dense granules proteins to facilitate secretion of transmembrane-domain containing proteins (Braun *et al.*, 2008). Therefore, the function of the NcROP2Fam-1, NcGRA7 and NcNTPase phosphorylation at the egress stage awaits further study.

Remarkably, several studied rhoptry proteins in *T. gondii* have been described as kinases or pseudokinases, and some of them have shown the ability to remodel cellular transduction and the transcriptome of the host cell through phosphorylation events (Reese *et al.*, 2014; Jacot & Soldati-Favre, 2012; Lim *et al.*, 2012). NcROP40 and NcROP2Fam-1 display high sequence similarity with the TgROP5, which also lacks kinase activity (Talevich & Kannan, 2013). Similarly to NcROP2Fam-1, the TgROP5 protein is clearly secreted during invasion and associates with the PVM (Reese & Boothroyd, 2011; El Hajj *et al.*, 2007b). Interestingly, TgROP5, TgROP18, and TgROP17 form complexes that inactivate host immune responses and inflammation (Du *et al.*, 2014; Etheridge *et al.*, 2014). Thus, a similar function would be expected for the NcROP2Fam-1 protein. Unfortunately, little is known about the existence of rhoptry virulence factors that could alter the host transcriptome after the infection with *N. caninum* (Reid *et al.*, 2012).

In summary, the second objective of the present thesis describes highly interesting features of the NcROP40, NcROP2Fam-1, NcGRA7 and NcNTPase during the lytic cycle of *N. caninum* tachyzoites. Our results can be taken as a first approach to increase the limited existing knowledge about these proteins in *N. caninum*, and serve as reference for future studies intended to establish their functional role. However, the improvement of the current gene annotation within the Nc-Liv genome and the incorporation of new fully sequenced isolates would be highly desirable. In this sense, the development of new reverse genetic tools applied to *N. caninum* appears to be a valuable approach to determine the functional role of NcROP40, NcROP2Fam-1, NcGRA7 and NcNTPase during the tachyzoite lytic cycle and their putative implications in parasite virulence.

The third objective of the present doctoral thesis aimed to evaluate the usefulness of the NcROP40, NcROP2Fam-1, NcNTPase, and NcGRA7 proteins formulated as subunit vaccines to prevent *N. caninum* infection in a well-established mouse model. Herein, we hypothesized that vaccines against components of rhoptries or dense granules could elicit an effective immune response to block the tachyzoite lytic cycle. Consequently, NcROP40 and NcNTPase were selected as vaccine targets due to their potential role in parasite virulence (Regidor-Cerrillo *et al.*, 2012), whereas NcROP2Fam-1 and NcGRA7 were chosen due to their immunogenicity and effectiveness as vaccines evidenced in previous trials (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a; Debache *et al.*, 2009). All the proteins were tested as monolovalent and polyvalent vaccines. Polyvalent vaccines were formulated according to the predicted functional role and the likely association of each protein during the tachyzoite lytic cycle (Cesbron-Delauw *et al.*, 2008; El Hajj *et al.*, 2006; Ferguson, 2004; Coppens *et al.*, 1999). Quil-A was chosen as an adjuvant to

elicit a balanced Th1/Th2 response and prevent the appearance of immunological disorders during pregnancy (Kano *et al.*, 2007; Chaouat *et al.*, 1990).

After inoculation, vaccine safety was exhaustively monitored. For this purpose, a detailed and objective scoring system was established here for the first time, appearing as a valuable tool to standardise the results from future vaccination trials. None of the vaccine formulations caused severe adverse effects. However, mice vaccinated with rNcGRA7+rNcNTPase displayed the worst safety scores due to a stronger local response at the point of inoculation. Despite the fact that on bacterially expressed antigens conformational epitopes may not be exposed, Western-blot and ELISA tests demonstrated the immunogenicity of all the inoculated antigens. Mice developed antibodies against each recombinant protein, and these antibodies also recognized their respective native proteins in tachyzoite extracts. In addition, this response remained active after the infection, since sera from vaccinated and challenged animals recognized the same specific bands. Hence, the lack of a protective effect cannot be attributed to a lack of immune stimulation against the original protein as reported earlier for NcSAG1, NcSRS2 and NcSAG4 (Aguado-Martínez *et al.*, 2009a; Cannas *et al.*, 2003a). The marked differences between IgG1 and IgG2a levels in the mice vaccinated with rNcROP40+rNcROP2Fam-1 did not alter the pregnancy of dams. In contrast, those mice immunized with rNcGRA7+rNcNTPase displayed the lowest fertility rate and the highest early pup mortality, which may be related to an alteration of the Th1/Th2 balance as previously suggested (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a). Nevertheless, the high degree of immunological stimulation of this group could have been detrimental to the pregnancy establishment during the mating phase.

Vaccination with rNcGRA7, rNcSAG4, rNcSRS9 and rNcBSR4 in the same animal model led to higher rates of morbidity and mortality, with the appearance of neurological symptoms in many cases (Jiménez-Ruiz *et al.*, 2012; Aguado-Martínez *et al.*, 2009a). In contrast, our results point towards a reduction of the severity of *N. caninum* infection in all vaccinated mice, which displayed low morbidity scores and mortality rates. According to this, vaccination of mice with rNcROP2Fam-1 also protected against clinical signs of neosporosis in previous studies (Debache *et al.*, 2010; Debache *et al.*, 2009; Debache *et al.*, 2008). On the other hand, dams vaccinated with rhoptry proteins exhibited the lowest cerebral parasite burdens, while vaccination with dense granule proteins resulted in the highest ones. In the same way, the frequency of parasite detection in brains was lower in dams vaccinated with rhoptry proteins. Although these findings were not statistically significant, they could reflect a residual impediment of parasite replication due to vaccination with rhoptry proteins. According to this, IgG responses were significantly higher in mice vaccinated with rNcGRA7 and rNcGRA7+rNcNTPase and lower in mice inoculated with rhoptry proteins. These results could be explained by means of the immunodominant role of NcGRA7, as mentioned above, or by a lower stimulation of the immune cells in those groups immunized with rhoptry proteins due to a lower number of replicating parasites.

Vaccination with rNcROP40+rNcROP2Fam-1, rNcROP40, rNcROP2Fam-1, rNcGRA7 and rNcNTPase slightly prolonged the median survival time of pups, but only rNcROP2Fam-1 and rNcROP40+rNcROP2Fam-1 prevented pup mortality, at least partially, with survival rates of 6.3 and 16.2%, respectively. Remarkably, the rNcROP40+rNcROP2Fam-1 combination showed a synergistic effect and prevented vertical transmission in all surviving pups born from different litters. This is in contrast with the 100% of vertical transmission previously described by vaccinating with rNcROP2Fam-1, despite the higher survival rates (Debache *et al.*, 2009). However, these differences could be attributed to the use of different isolates for challenge infection (Regidor-Cerrillo *et al.*, 2010a). Furthermore, the variable protection observed could also be due to the use of different adjuvants as previously demonstrated (Rojo-Montejo *et al.*, 2011b). Conversely, no synergism was achieved by combining rNcGRA7 and rNcNTPase. Previous studies described a potentiated effect against cyst formation in *T. gondii* after vaccination with rTgROP2+rTgROP4, which was not observed after single inoculation of these proteins (Dziadek *et al.*, 2012; Dziadek *et al.*, 2011; Dziadek *et al.*, 2009). In contrast, vaccination with rTgROP2, rTgGRA4 and rTgROP2+rTgGRA4 combination vaccine against *T. gondii* challenge infection in mice exhibited the same efficacy (Sanchez *et al.*, 2011), supporting our findings concerning the synergistic effect, but only in groups vaccinated with rhoptry proteins.

The secretion of IFN-γ has been associated with the control of parasite dissemination and congenital transmission of *N. caninum* in mouse models (Kano *et al.*, 2005; Quinn *et al.*, 2004; Long *et al.*, 1998). In our experiments, vaccination with rNcROP2Fam-1 induced the highest levels of IFN-γ secretion. Besides this, the groups vaccinated with rNcROP40 and rNcROP40+rNcROP2Fam-1 secreted higher levels of IFN-γ than those vaccinated with proteins from dense granules. However, higher levels of IFN-γ secretion did not correspond to the best results in terms of pup survival. rNcROP40+rNcROP2Fam-1-vaccinated mice registered the highest pup survival rate (16.2%) with low levels of IFN-γ secretion in stimulated splenocytes. In addition, vaccination had a positive effect on median survival time in the majority of the groups regardless of the secreted levels of IFN-γ. Interestingly, IgG1 levels remained high during the induction and effector phases of the trial in most of the groups, which support the fact that humoral responses also play a key role in the protection against *N. caninum* infection, as previously demonstrated with both recombinant and live vaccines (Marugán-Hernández *et al.*, 2011b; Haldorson *et al.*, 2005).

In summary, rNcROP40 and rNcROP2Fam-1 inoculation resulted in a partial block of transplacental transmission under a synergistic effect. Therefore, the use of rhoptry proteins as antigens in vaccine formulations appears as a novel strategy to block the processes in which they are involved. The present study takes into account the worst scenario of neosporosis, since the BALB/c model employed here ensures a 100% congenital transmission and 0% of survival of the offspring in the non-vaccinated mice (López-Pérez *et al.*, 2008). Hence, despite the seemingly low level of protection achieved herein, our results are encouraging. Moreover, we propose an objective scoring system to evaluate vaccine safety and

efficacy that will enable future study comparisons. Indeed, several factors such as the inbred strains of mice, the mouse model (pregnant or non-pregnant), and the type and dose of isolate used for challenge determine the outcome of the infection and limit the ability to compare the results from different studies (Dellarupe *et al.*, 2014a; Monney & Hemphill, 2014; Mols-Vorstermans *et al.*, 2013; Regidor-Cerrillo *et al.*, 2010a; Collantes-Fernández *et al.*, 2006b). In addition, it has been recently shown that most vaccine trials have been carried out with an exceedingly high challenge dose, and a reduced parasite challenge dose has been proposed for future studies (Arranz-Solís *et al.*, 2015a). Taken together, these findings highlight the urgent need to adopt a common mouse model that allows a valid comparison of results from different research groups.

CONCLUSIONES/CONCLUSIONS

Objetivo 1: Determinación de los cambios en el inmunoma y en el proteoma de aislados de *N. caninum* de alta y baja virulencia.

Sub-objetivo 1.1: Estudio de los cambios en el proteoma.

Primera: Los aislados virulentos Nc-Liverpool y Nc-Spain7, así como el aislado no virulento Nc-Spain1H, presentan variaciones relevantes en la abundancia de sus proteínas. El aislado Nc-Spain7 presentó el mayor número de manchas proteicas diferencialmente abundantes frente a los aislados Nc-Liverpool y Nc-Spain1H. No obstante, no fue posible identificar ninguna mancha proteica específica en ninguno de los aislados estudiados.

Segunda: Se han identificado un total de once proteínas con diferente abundancia al comparar aislados virulentos y no virulentos. Estas proteínas están potencialmente implicadas en la motilidad, el ciclo lítico del taquizoíto, el estrés oxidativo y otras funciones diversas. El estudio de estas proteínas permitiría avanzar en el conocimiento de los mecanismos implicados en la virulencia de *N. caninum*.

Sub-Objetivo 1.2: Estudio de las diferencias en el inmunoma.

Primera: Las diferencias observadas en el inmunoma dependen exclusivamente de la respuesta inmunitaria inducida por cada uno de los aislados estudiados, y no del extracto proteico empleado. Asimismo, se ha observado la presencia de un patrón jerárquico de reconocimiento antigénico entre los aislados virulentos y no virulentos, que podría estar determinado por la dinámica de la infección de cada uno de ellos en el ratón. De acuerdo con esto, los sueros de los ratones infectados con aislados virulentos reconocieron el mayor número de antígenos, mientras que los sueros de los ratones infectados con el aislado no virulento reaccionaron frente a un menor número de antígenos.

Segunda: Diversas proteínas relacionadas con el metabolismo, asociadas a la motilidad y a la invasión de los taquizoítos y situadas en gránulos densos, no fueron detectadas por los sueros de los ratones infectados con el aislado de baja virulencia Nc-Spain1H, por lo que podrían considerarse como marcadores potenciales de virulencia.

Objetivo 2: Descripción y caracterización de las proteínas NcROP40 y NcNTPasa a lo largo del ciclo lítico del taquizoíto mediante abordajes *in silico* e *in vitro*.

Sub-Objetivo 2.1: Caracterización de las proteínas de roptrias NcROP40 y NcROP2Fam-1 de *N. caninum*.

Primera: Las pseudoquinasas NcROP40 y NcROP2Fam-1 muestran características dispares a lo largo del ciclo lítico, lo que sugiere que pueden llevar a cabo funciones diferentes en el mismo. Ambas proteínas se localizan en el bulbo de las roptrias, pero en comparación con NcROP2Fam-1, NcROP40 no se asocia con la membrana de la vacuola parasitófora ni se detecta en evacuolas. No obstante, no se descarta que NcROP40 pueda secretarse en el citosol y/o en el núcleo de la célula hospedadora. Por otro lado, la secreción de NcROP2Fam-1 sólo se induce tras el contacto del parásito con la célula hospedadora, por lo que es de esperar que la posible secreción de NcROP40 tenga un comportamiento similar.

Segunda: Los mecanismos de regulación de NcROP40 parecen ser diferentes a los de NcROP2Fam-1 a lo largo del ciclo lítico. En este sentido, es probable que la expresión de transcritos de NcROP40 no esté vinculada con los mecanismos de egresión, aunque su función podría ser relevante para las fases posteriores sin que sea necesario que la proteína esté fosforilada. Por el contrario, NcROP2Fam-1 parece ser necesaria para la egresión y la invasión; su fosforilación podría regular la actividad de la proteína durante estos procesos.

Sub-Objetivo 2.2: Caracterización de las proteínas de gránulos densos NcNTPasa y NcGRA7 de *N. caninum*.

Primera: La proteína NcNTPasa se encuentra codificada por tres *loci* diferentes en el genoma del aislado Nc-Liverpool. Éstos contienen hasta seis alelos distintos que podrían codificar las diversas especies de las proteínas detectadas. De acuerdo con estos hallazgos, las variaciones en el número de alelos y en el patrón de expresión de la NcNTPasa podrían ser la causa de las diferentes abundancias de esta proteína entre los aislados virulentos y no virulentos de *N. caninum*.

Segunda: Las proteínas de gránulos densos NcNTPasa y NcGRA7, que comparten un perfil transcriptómico similar, podrían ser necesarias para la egresión y la invasión tras su fosforilación. Ambas proteínas se secretan a lo largo del ciclo lítico e interactúan con la membrana de la vacuola parasitófora. Sin embargo, su distribución temporal dentro del taquizoíto es diferente durante las fases de egresión e invasión. Además, en contraste con NcGRA7, la secreción de la proteína NcNTPasa es independiente de las rutas de señalización mediadas por el calcio implicadas en la egresión y la secreción de micronemas durante la invasión.

Objetivo 3: Evaluación de NcROP40 y NcNTPasa como candidatos vacunales en formulaciones monovalentes y polivalentes combinadas con las proteínas NcROP2Fam-1 y NcGRA7 en un modelo murino de neosporosis.

Primera: La formulaciones vacunales basadas en las proteínas rNcROP40, rNcROP2Fam-1, rNcGRA7, rNcNTPasa y sus combinaciones son seguras. En este sentido, el empleo de un sistema de valoración de los signos clínicos exhaustivo y objetivo para la evaluación de la seguridad y eficacia de la vacuna, se presenta como una herramienta útil que posibilita la comparación de los resultados obtenidos por diferentes grupos de investigación.

Segunda: La combinación de proteínas de roptrias para el desarrollo de vacunas de subunidades frente a la neosporosis resulta prometedora. Pese a que la inmunización con cada uno de los candidatos vacunales o sus combinaciones se asoció con una respuesta inmunitaria específica frente a sus respectivas proteínas nativas, únicamente, las formulaciones basadas en la proteína rNcROP2Fam-1 y en la mezcla de rNcROP40 con rNcROP2Fam-1 protegieron, al menos parcialmente, frente a la mortalidad de las crías, alcanzando tasas de supervivencia del 6,3 y 16,2%, respectivamente. Además, la combinación de las proteínas rNcROP40 y rNcROP2Fam-1 tuvo un efecto sinérgico protegiendo frente a la transmisión vertical en todas las crías supervivientes, procedentes de diferentes camadas.

Objective 1: determination of the proteome and immunome changes among *N. caninum* virulent and attenuated isolates

Sub-objective 1.1: Study of the proteome expression changes.

First: There are marked variations in protein abundance among the virulent Nc-Liverpool and Nc-Spain7 isolates, and the non-virulent Nc-Spain1H isolate. The Nc-Spain7 isolate showed the highest number of differentially abundant spots with respect to Nc-Liv and Nc-Spain1H isolates. However, no specific-isolate spots were found.

Second: A total of eleven proteins potentially involved in gliding motility, the tachyzoite lytic cycle, oxidative stress, and miscellaneous functions were differentially abundant among virulent and non-virulent isolates. Therefore, the study of the mentioned proteins could provide a better knowledge of the mechanisms involved in the parasite virulence in *N. caninum*.

Sub-objective 1.2: Study of the immunome changes.

First: Differences in the immunome profiles relied on the immune response induced by each isolate, regardless of the antigenic extract. This study reveals a hierarchical pattern of antigen recognition among virulent and non-virulent isolates, which may be associated with the infection dynamics in mice. According to this, sera from mice infected with high virulence isolates recognized the highest number of antigens, whilst sera from mice infected with low virulence isolates recognized less antigens.

Second: Diverse proteins related to metabolism and tachyzoites invasion, and located on dense granules, failed to be detected by sera from mice infected with the low virulence isolate Nc-Spain1H. Therefore, these proteins emerge as potential virulence markers for bovine neosporosis.

Objective 2: description and characterization of the NcROP40 and NcNTPase proteins along the tachyzoite lytic cycle through *in silico* and *in vitro* approaches

Sub-objective 2.1: Characterization of the *N. caninum* NcROP40 and NcROP2Fam-1 rhoptry proteins.

First: NcROP40 and NcROP2Fam-1 pseudokinases display different features throughout the tachyzoite lytic cycle, suggesting that they may carry out different functions therein. Both proteins are localized within the rhoptry bulbs, but in contrast to NcROP2Fam-1, NcROP40 did not associate with the parasitophorous vacuole membrane and was not detected into the host cell forming evacuoles. However, NcROP40 secretion into the host cytosol and/or nucleus was not discarded. NcROP2Fam-1 discharge was only induced with success upon host cell contact, and similar findings would be expected for the NcROP40 protein.

Second: NcROP40 may be subjected to different regulation mechanisms compared with NcROP2Fam-1 throughout the lytic cycle. Specifically, the mRNA expression of NcROP40 was not apparently linked with the mechanisms triggering egress, but its function may be relevant for the subsequent phases of the cycle and independent of its phosphorylation. Oppositely, NcROP2Fam-1 seems necessary for both egress and invasion, and protein phosphorylation could regulate the protein activity during these processes.

Sub-objective 2.2: Characterization of the *N. caninum* NcNTPase and NcGRA7 dense granule proteins.

First: The NcNTPase protein is coded by three different *loci* within the Nc-Liverpool genome. Moreover, the occurrence of up to six different Nc*NTPase* alleles and diverse protein species has been also evidenced. Variations in the pattern of expression and number of alleles for each NcNTPase *loci* could explain the differences in expression of the protein among *N. caninum* isolates.

Second: The dense granule NcNTPase and NcGRA7 proteins share similar transcriptional profiles, and could be necessary for both egress and invasion after their phosphorylation. Both proteins were secreted throughout the lytic cycle and interact with the parasitophorous vacuole membrane. However, they displayed a distinct temporal distribution within the tachyzoite during invasion and egress phases. Furthermore, and in contrast to NcGRA7, NcNTPase secretion occurred in tachyzoites regardless of the calcium signalling cascades and the microneme secretion.

Objective 3: evaluation of the usefulness of NcROP40 and NcNTPase-based vaccines in single and polyvalent formulations combined with the NcROP2Fam-1 and NcGRA7 proteins in a mouse model of neosporosis.

First: Vaccine formulations based on rNcROP40, rNcROP2Fam-1, rNcGRA7, rNcNTPase, and their combinations have proven to be safe. The establishment of an exhaustive and objective scoring system to evaluate both vaccine safety and clinical signs after parasite challenge appears as a useful tool to adopt a common mouse model that allows a valid comparison of results from different research groups.

Second: Rhoptry combinations may be the base of new promising subunit vaccines against neosporosis. The immunization with all the vaccine candidates and their combinations elicited a specific immune response against their respective native proteins. However, only rNcROP2Fam-1 and rNcROP40+rNcROP2Fam-1 formulations partially prevented pup mortality with survival rates of 6.3 and 16.2%, respectively. Moreover, rNcROP40 and rNcROP2Fam-1 combination showed a synergistic effect and blocked vertical transmission in all surviving pups from different litters.

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